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(54) Non-human Carbonyl hydrolase mutants, DNA sequences and vectors encoding same and hosts transformed with said vectors.

(57) Novel carbonyl hydrolase mutants derived from the amino acid sequence of naturally-occurring or recombinant non-human carbonyl hydrolases and DNA sequences encoding the same. The mutant carbonyl hydrolases, in general, are obtained by *in vitro* modification of a precursor DNA sequence encoding the naturally-occurring or recombinant carbonyl hydrolase to encode the substitution, insertion or deletion of one or more amino acids in the amino acid sequence of a precursor carbonyl hydrolase. Such mutants have one or more properties which are different than the same property of the precursor hydrolase.

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NON-HUMAN CARBONYL HYDROLASE MUTANTS,
DNA SEQUENCES AND VECTORS ENCODING SAME
AND HOSTS TRANSFORMED WITH SAID VECTORS

The recent development of various in vitro techniques to manipulate the DNA sequences encoding naturally-occurring polypeptides as well as recent developments in the chemical synthesis of relatively short sequences of single and double stranded DNA has resulted in the speculation that such techniques can be used to modify enzymes to improve some functional property in a predictable way. Ulmer, K.M. (1983) Science 219, 666-671. The only working example disclosed therein is the substitution of a single amino acid within the active site of tyrosyl-tRNA synthetase (Cys35-Ser) which lead to a reduction in enzymatic activity. See Winter, G., et al. (1982) Nature 299, 756-758; and Wilkinson, A.J., et al. (1983) Biochemistry 22, 3581-3586 (Cys35-Gly mutation also resulted in decreased activity).

When the same t-RNA synthetase was modified by substituting a different amino acid residue within the active site with two different amino acids, one of the mutants (Thr51-Ala) reportedly demonstrated a predicted moderate increase in k_{cat}/K_m whereas a second mutant (Thr51-Pro) demonstrated a massive increase in k_{cat}/K_m which could not be explained with

certainty. Wilkinson, A.H., et al. (1984) Nature 307, 187-188.

5 Another reported example of a single substitution of
an amino acid residue is the substitution of cysteine
for isoleucine at the third residue of T4 lysozyme.
Perry, L.J., et al. (1984) Science 226, 555-557. The
resultant mutant lysozyme was mildly oxidized to form
a disulfide bond between the new cysteine residue at
position 3 and the native cysteine at position 97.
10 This crosslinked mutant was initially described by the
author as being enzymatically identical to, but more
thermally stable than, the wild type enzyme. However,
in a "Note Added in Proof", the author indicated that
the enhanced stability observed was probably due to a
15 chemical modification of cysteine at residue 54 since
the mutant lysozyme with a free thiol at Cys54 has a
thermal stability identical to the wild type lysozyme.

20 Similarly, a modified dihydrofolate reductase from
E.coli has been reported to be modified by similar
methods to introduce a cysteine which could be
crosslinked with a naturally-occurring cysteine in the
reductase. Villafranca, D.E., et al. (1983) Science
222, 782-788. The author indicates that this mutant
25 is fully reactive in the reduced state but has
significantly diminished activity in the oxidized
state. In addition, two other substitutions of
specific amino acid residues are reported which
resulted in mutants which had diminished or no
30 activity.

EPO Publication No. 0130756 discloses the substitution
of specific residues within B. amyloliquefaciens
subtilisin with specific amino acids. Thus, Met222
35 has been substituted with all 19 other amino acids,

Gly166 with 9 different amino acids and Gly169 with Ala and Ser.

As set forth below, several laboratories have also reported the use of site directed mutagenesis to produce the mutation of more than one amino acid residue within a polypeptide.

The amino-terminal region of the signal peptide of the prolipoprotein of the E. coli outer membrane was stated to be altered by the substitution or deletion of residues 2 and 3 to produce a charge change in that region of the polypeptide. Inouye, S., et al. (1982) Proc. Nat. Acad. Sci. USA 79, 3438-3441. The same laboratory also reported the substitution and deletion of amino acid residues 9 and 14 to determine the effects of such substitution on the hydrophobic region of the same signal sequence. Inouye, S., et al. (1984) J. Biol. Chem. 259, 3729-3733.

Double mutants in the active site of tyrosyl-t-RNA synthetase have also been reported. Carter, P.J., et al. (1984) Cell 38, 835-840. In this report, the improved affinity of the previously described Thr51-Pro mutant for ATP was probed by producing a second mutation in the active site of the enzyme. One of the double mutants, Gly35/Pro51, reportedly demonstrated an unexpected result in that it bound ATP in the transition state better than was expected from the two single mutants. Moreover, the author warns, at least for one double mutant, that it is not readily predictable how one substitution alters the effect caused by the other substitution and that care must be taken in interpreting such substitutions.

A mutant is disclosed in U.S. Patent No. 4,532,207, wherein a polyarginine tail was attached to the C-terminal residue of β -urogastrone by modifying the DNA sequence encoding the polypeptide. As disclosed, the polyarginine tail changed the electrophoretic mobility of the urogastrone-polyarginine hybrid permitting selective purification. The polyarginine was subsequently removed, according to the patentee, by a polyarginine specific exopeptidase to produce the purified urogastrone. Properly construed, this reference discloses hybrid polypeptides which do not constitute mutant polypeptides containing the substitution, insertion or deletion of one or more amino acids of a naturally occurring polypeptide.

Single and double mutants of rat pancreatic trypsin have also been reported. Craik, C.S., et al. (1985) Science 228, 291-297. As reported, glycine residues at positions 216 and 226 were replaced with alanine residues to produce three trypsin mutants (two single mutants and one double mutant). In the case of the single mutants, the authors stated expectation was to observe a differential effect on K_m . They instead reported a change in specificity (k_{cat}/K_m) which was primarily the result of a decrease in k_{cat} . In contrast, the double mutant reportedly demonstrated a differential increase in K_m for lysyl and arginyl substrates as compared to wild type trypsin but had virtually no catalytic activity.

The references discussed above are provided solely for their disclosure prior to the filing date of the instant case, and nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention or priority based on earlier filed applications.

Based on the above references, however, it is apparent that the modification of the amino acid sequence of wild type enzymes often results in the decrease or destruction of biological activity.

5 Accordingly, it is an object herein to provide carbonyl hydrolase mutants which have at least one property which is different from the same property of the carbonyl hydrolase precursor from which the amino acid of said mutant is derived.

10 It is a further object to provide mutant DNA sequences encoding such carbonyl hydrolase mutants as well as expression vectors containing such mutant DNA sequences.

15 Still further, another object of the present invention is to provide host cells transformed with such vectors as well as host cells which are capable of expressing such mutants either intracellularly or
20 extracellularly.

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Summary of the Invention

The invention includes carbonyl hydrolase mutants, preferably having at least one property which is substantially different from the same property of the precursor non-human carbonyl hydrolase from which the amino acid sequence of the mutant is derived. These properties include oxidative stability, substrate, specificity catalytic activity, thermal stability, alkaline stability, pH activity profile and resistance to proteolytic degradation. The precursor carbonyl hydrolase may be naturally occurring carbonyl hydrolases or recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is derived by the substitution, deletion or insertion of one or more amino acids of the precursor carbonyl hydrolase amino acid sequence.

The invention also includes mutant DNA sequences encoding such carbonyl hydrolase mutants. Further the invention includes expression vectors containing such mutant DNA sequences as well as host cells transformed with such vectors which are capable of expressing said carbonyl hydrolase mutants.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of the coding strand, correlated with the amino acid sequence of B. amyloliquefaciens subtilisin gene. Promoter (p) ribosome binding site (rbs) and termination (term) regions of the DNA sequence as well as sequences encoding the presequence (PRE) putative prosequence (PRO) and mature form (MAT) of the hydrolase are also shown.

Figure 2 is a schematic diagram showing the substrate binding cleft of subtilisin together with substrate.

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Figure 3 is a stereo view of the S-1 binding subsite of B. amyloliquefaciens subtilisin showing a lysine P-1 substrate bound in the site in two different ways. Figure 3A shows Lysine P-1 substrate bound to form a salt bridge with a Glu at position 156. Figure 3B shows Lysine P-1 substrate bound to form a salt bridge with Glu at position 166.

Figure 4 is a schematic diagram of the active site of subtilisin Asp32, His64 and Ser221.

Figures 5A and 5B depict the amino acid sequence of subtilisin obtained from various sources. The residues directly beneath each residue of B. amyloliquefaciens subtilisin are equivalent residues which (1) can be mutated in a similar manner to that described for B. amyloliquefaciens subtilisin, or (2) can be used as a replacement amino acid residue in B. amyloliquefaciens subtilisin. Figure 5C depicts conserved residues of B. amyloliquefaciens subtilisin when compared to other subtilisin sequences.

Figures 6A and 6B depict the inactivation of the mutants Met222L and Met222Q when exposed to various organic oxidants.

Figure 7 depicts the ultraviolet spectrum of Met222F subtilisin and the difference spectrum generated after inactivation by dimerdodecanoic acid (DPDA).

Figure 8 shows the pattern of cyanogen bromide digests of untreated and DPDA oxidized subtilisin Met222F on high resolution SDS-pyridine peptide gels.

Figure 9 depicts a map of the cyanogen bromide fragments of Fig. 8 and their alignment with the sequence of subtilisin Met222F.

5 Figure 10 depicts the construction of mutations between codons 45 and 50 of B. amyloliquefaciens subtilisin.

10 Figure 11 depicts the construction of mutations between codons 122 and 127 of B. amyloliquefaciens subtilisin.

15 Figure 12 depicts the effect of DPDA on the activity of subtilisin mutants at positions 50 and 124 in subtilisin Met222F.

Figure 13 depicts the construction of mutations at codon 166 of B. amyloliquefaciens subtilisin.

20 Figure 14 depicts the effect of hydrophobicity of the P-1 substrate side-chain on the kinetic parameters of wild-type B. amyloliquefaciens subtilisin.

25 Figure 15 depicts the effect of position 166 side-chain substitutions on P-1 substrate specificity. Figure 15A shows position 166 mutant subtilisins containing non-branched alkyl and aromatic side-chain substitutions arranged in order of increasing molecular volume. Figure 15B shows a series of mutant enzymes progressing through β - and γ -branched aliphatic side chain substitutions of increasing molecular volume.

30 Figure 16 depicts the effect of position 166 side-chain volume on log k_{cat}/K_m for various P-1 substrates.

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Figure 17 shows the substrate specificity differences between Ile166 and wild-type (Gly166) B. amyloliquefaciens subtilisin against a series of alphatic and aromatic substrates. Each bar represents the difference in log kcat/Km for Ile166 minus wild-type (Gly166) subtilisin.

Figure 18 depicts the construction of mutations at codon 169 of B. amyloliquefaciens subtilisin.

Figure 19 depicts the construction of mutations at codon 104 of B. amyloliquefaciens subtilisin.

Figure 20 depicts the construction of mutations at codon 152 B. amyloliquefaciens subtilisin.

Figure 21 depicts the construction of single mutations at codon 156 and double mutations at codons 156 and 166 of B. amyloliquefaciens subtilisin.

Figure 22 depicts the construction of mutations at codon 217 for B. amyloliquefaciens subtilisin.

Figure 23 depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 23A depicts the kcat/Km versus pH profile for mutations at codon 156 and 166 in B. amyloliquefaciens subtilisin.

Figure 24 depicts the kcat/Km versus pH profile for mutations at codon 222 in B. amyloliquefaciens subtilisin.

Figure 25 depicts the constructing mutants at codons 94, 95 and 96.

Figures 26 and 27 depict substrate specificity of various wild type and mutant subtilisins for different substrates.

Figures 28 A, B, C and D depict the effect of charge in the P-1 binding sites due to substitutions at codon 156 and 166.

Figures 29 A and B are a stereoview of the P-1 binding site of subtilisin BPN' showing a lysine P-1 substrate bound in the site in two ways. In 29A, Lysine P-1 substrate is built to form a salt bridge with a Glu at codon 156. In 29B, Lysine P-1 substrate is built to form a salt bridge with Glu at codon 166.

Figure 30 demonstrates residual enzyme activity versus temperature curves for purified wild-type (Panel A), C22/C87 (Panel B) and C24/C87 (Panel C).

Figure 31 depicts the strategy for producing point mutations in the subtilisin coding sequence by misincorporation of α -thioldeoxynucleotide triphosphates.

Figure 32 depicts the autolytic stability of purified wild type and mutant subtilisins 170E, 107V, 213R and 107V/213R at alkaline pH.

Figure 33 depicts the autolytic stability of purified wild type and mutant subtilisins V50, F50 and F50/V107/R213 at alkaline pH.

Figure 34 depicts the strategy for constructing plasmids containing random cassette mutagenesis over residues 197 through 228.

5 Figure 35 depicts the oligodeoxynucleotides used for random cassette mutagenesis over residues 197 through 228.

10 Figure 36 depicts the construction of mutants at codon 204.

Figure 37 depicts the oligodeoxynucleotides used for synthesizing mutants at codon 204.

15 Detailed Description

The inventors have discovered that various single and multiple in vitro mutations involving the substitution, deletion or insertion of one or more amino acids within a non-human carbonyl hydrolase amino acid sequence can confer advantageous properties to such mutants when compared to the non-mutated carbonyl hydrolase.

20 Specifically, B. amyloliquefaciens subtilisin, an alkaline bacterial protease, has been mutated by modifying the DNA encoding the subtilisin to encode the substitution of one or more amino acids at various amino acid residues within the mature form of the subtilisin molecule. These in vitro mutant subtilisins have at least one property which is different when compared to the same property of the precursor subtilisin. These modified properties fall into several categories including: oxidative stability, substrate specificity, thermal stability, alkaline stability, catalytic activity, pH activity

profile, resistance to proteolytic degradation, K_m , k_{cat} and K_m/k_{cat} ratio.

Carbonyl hydrolases are enzymes which hydrolyze

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5 compounds containing C-X bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally occurring carbonyl hydrolases principally
10 include hydrolases, e.g. lipases and peptide hydrolases, e.g. subtilisins or metalloproteases. Peptide hydrolases include α -aminoacylpeptide hydrolase, peptidylamino-acid hydrolase, acylamino
15 hydrolase, serine carboxypeptidase, metallo-carboxypeptidase, thiol proteinase, carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

20 "Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the
25 substitution, insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein and in EPO Publication No. 0130756 published January 9, 1985.

30 Subtilisins are bacterial carbonyl hydrolases which generally act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally occurring subtilisin or a recombinant subtilisin. A series of naturally occurring
35 subtilisins is known to be produced and often secreted

by various bacterial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related proteases the relative order of these amino acids, reading from the amino to carboxy terminus is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however is histidine-aspartate-serine. Thus, subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases.

"Recombinant subtilisin" refers to a subtilisin in which the DNA sequence encoding the subtilisin is modified to produce a mutant DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally occurring subtilisin amino acid sequence. Suitable methods to produce such modification include those disclosed herein and in EPO Publication No. 0130756. For example, the subtilisin multiple mutant herein containing the substitution of methionine at amino acid residues 50, 124 and 222 with phenylalanine, isoleucine and glutamine, respectively, can be considered to be derived from the recombinant subtilisin containing the substitution of glutamine at residue 222 (Q222) disclosed in EPO Publication No. 0130756. The multiple mutant thus is produced by the substitution of phenylalanine for methionine at

residue 50 and isoleucine for methionine at residue 124 in the Q222 recombinant subtilisin.

"Carbonyl hydrolases" and their genes may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *pseudomonas* and gram positive bacteria such as *micrococcus* or *bacillus*. Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *S. cerevisiae*, fungi such as *Aspergillus* sp., and non-human mammalian sources such as, for example, Bovine sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or indirectly, with procaryotic and non-human eucaryotic sources.

A "carbonyl hydrolase mutant" has an amino acid sequence which is derived from the amino acid sequence of a non-human "precursor carbonyl hydrolase". The precursor carbonyl hydrolases include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. The amino acid sequence of the carbonyl hydrolase mutant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the

5 amino acid sequence of the precursor carbonyl
hydrolase rather than manipulation of the precursor
carbonyl hydrolase per se. Suitable methods for such
manipulation of the precursor DNA sequence include
methods disclosed herein and in EPO Publication No.
0130756.

10 Specific residues of B. amyloliquefaciens subtilisin
are identified for substitution, insertion or
deletion. These amino acid position numbers refer to
those assigned to the B. amyloliquefaciens subtilisin
sequence presented in Fig. 1. The invention, however,
is not limited to the mutation of this particular
subtilisin but extends to precursor carbonyl
15 hydrolases containing amino acid residues which are
"equivalent" to the particular identified residues in
B. amyloliquefaciens subtilisin.

20 A residue (amino acid) of a precursor carbonyl
hydrolase is equivalent to a residue of B.
amyloliquefaciens subtilisin if it is either
homologous (i.e., corresponding in position in either
primary or tertiary structure) or analagous to a
specific residue or portion of that residue in B.
25 amyloliquefaciens subtilisin (i.e., having the same or
similar functional capacity to combine, react, or
interact chemically).

30 In order to establish homology to primary structure,
the amino acid sequence of a precursor carbonyl
hydrolase is directly compared to the B.
amyloliquefaciens subtilisin primary sequence and
particularly to a set of residues known to be
invariant in all subtilisins for which sequence is
known (Figure 5C). After aligning the conserved
35 residues, allowing for necessary insertions and

deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of *B. amyloliquefaciens* subtilisin are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

For example, in Figure 5A the amino acid sequence of subtilisin from *B. amyloliquefaciens* *B. subtilisin* var. I168 and *B. lichenformis* (carlsbergensis) are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in each sequence. These residues are identified in Fig. 5C.

These conserved residues thus may be used to define the corresponding equivalent amino acid residues of *B. amyloliquefaciens* subtilisin in other carbonyl hydrolases such as thermitase derived from *Thermoactinomyces*. These two particular sequences are aligned in Fig. 5B to produce the maximum homology of conserved residues. As can be seen there are a number of insertions and deletions in the thermitase sequence as compared to *B. amyloliquefaciens* subtilisin. Thus, in thermitase the equivalent amino acid of Tyr217 in *B. amyloliquefaciens* subtilisin is the particular lysine shown beneath Tyr217.

In Fig. 5A, the equivalent amino acid at position 217 in *B. amyloliquefaciens* subtilisin is Tyr. Likewise,

in B. subtilis subtilisin position 217 is also occupied by Tyr but in B. licheniformis position 217 is occupied by Leu.

Thus, these particular residues in thermitase, and subtilisin from B. subtilis and B. licheniformis may be substituted by a different amino acid to produce a mutant carbonyl hydrolase since they are equivalent in primary structure to Tyr217 in B. amyloliquefaciens subtilisin. Equivalent amino acids of course are not limited to those for Tyr217 but extend to any residue which is equivalent to a residue in B. amyloliquefaciens whether such residues are conserved or not.

Equivalent residues homologous at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography, are defined as those for which the atomic coordinates of 2 or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and B. amyloliquefaciens subtilisin (N on N, CA on CA, C on C, and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the B. amyloliquefaciens subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

Equivalent residues which are functionally analogous to a specific residue of B. amyloliquefaciens subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the B. amyloliquefaciens subtilisin as described herein. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography), which occupy an analogous position to the extent that although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of B. amyloliquefaciens subtilisin. The three dimensional structures would be aligned as outlined above.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a mutant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally occurring sequence. The carbonyl hydrolase mutants of the present invention include the mature forms of carbonyl hydrolase mutants as well as the pro- and prepro-forms of such hydrolase mutants. The prepro-forms are the preferred construction since

this facilitates the expression, secretion and maturation of the carbonyl hydrolase mutants.

5, "Expression vector" refers to a DNA construct
containing a DNA sequence which is operably linked to
a suitable control sequence capable of effecting the
expression of said DNA in a suitable host. Such
control sequences include a promoter to effect
transcription, an optional operator sequence to
control such transcription, a sequence encoding
suitable mRNA ribosome binding sites, and sequences
10 which control termination of transcription and
translation. The vector may be a plasmid, a phage
particle, or simply a potential genomic insert. Once
transformed into a suitable host, the vector may
replicate and function independently of the host
15 genome, or may, in some instances, integrate into the
genome itself. In the present specification,
"plasmid" and "vector" are sometimes used
interchangeably as the plasmid is the most commonly
used form of vector at present. However, the
20 invention is intended to include such other forms of
expression vectors which serve equivalent functions
and which are, or become, known in the art.

25 The "host cells" used in the present invention
generally are procaryotic or eucaryotic hosts which
preferably have been manipulated by the methods
disclosed in EPO Publication No. 0130756 to render

them incapable of secreting enzymatically active endoprotease. A preferred host cell for expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in enzymatically active neutral protease and alkaline protease (subtilisin). The construction of strain BG2036 is described in detail in EPO Publication No. 0130756 and further described by Yang, M.Y., et al. (1984) J. Bacteriol. 160, 15-21. Other host cells for expressing subtilisin include *Bacillus subtilis* I168 (EPO Publication No. 0130756).

Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the carbonyl hydrolase mutants or expressing the desired carbonyl hydrolase mutant. In the case of vectors which encode the pre or prepro form of the carbonyl hydrolase mutant, such mutants, when expressed, are typically secreted from the host cell into the host cell medium.

"Operably linked" when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the

general methods described herein in EPO Publication No. 0130756.

Once the carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in EPO Publication No. 0130756 and the production of carbonyl hydrolase mutants described herein.

The carbonyl hydrolase mutants of the present invention may be generated by site specific mutagenesis (Smith, M. (1985) Ann, Rev. Genet. 423; Zoeller, M.J., et al. (1982) Nucleic Acid Res. 10, 6487-6500), cassette mutagenesis (EPO Publication No. 0130756) or random mutagenesis (Shortle, D., et al. (1985) Genetics, 110, 539; Shortle, D., et al. (1986) Proteins: Structure, Function and Genetics, 1, 81; Shortle, D. (1986) J. Cell. Biochem, 30, 281; Alber, T., et al. (1985) Proc. Natl. Acad. of Sci., 82, 747; Matsumura, M., et al. (1985) J. Biochem., 260, 15298; Liao, H., et al. (1986) Proc. Natl. Acad. of Sci., 83 576) of the cloned precursor carbonyl hydrolase. Cassette mutagenesis and the random mutagenesis method disclosed herein are preferred.

The mutant carbonyl hydrolases expressed upon transformation of suitable hosts are screened for enzymes exhibiting one or more properties which are substantially different from the properties of the precursor carbonyl hydrolases, e.g., changes in substrate specificity, oxidative stability, thermal stability, alkaline stability, resistance to

proteolytic degradation, pH-activity profiles and the like.

5 A change in substrate specificity is defined as a difference between the k_{cat}/K_m ratio of the precursor carbonyl hydrolase and that of the hydrolase mutant. The k_{cat}/K_m ratio is a measure of catalytic efficiency. Carbonyl hydrolase mutants with increased or diminished k_{cat}/K_m ratios are described in the examples. Generally, the objective will be to secure
10 a mutant having a greater (numerically large) k_{cat}/K_m ratio for a given substrate, thereby enabling the use of the enzyme to more efficiently act on a target substrate. A substantial change in k_{cat}/K_m ratio is preferably at least 2-fold increase or decrease. However, smaller increases or decreases in the ratio
15 (e.g., at least 1.5-fold) are also considered substantial. An increase in k_{cat}/K_m ratio for one substrate may be accompanied by a reduction in k_{cat}/K_m ratio for another substrate. This is a shift in substrate specificity, and mutants exhibiting such
20 shifts have utility where the precursor hydrolase is undesirable, e.g. to prevent undesired hydrolysis of a particular substrate in an admixture of substrates. K_m and k_{cat} are measured in accord with known procedures, as described in EPO Publication No. 0130756 or as described herein.

Oxidative stability is measured either by known procedures or by the methods described hereinafter. A
30 substantial change in oxidative stability is evidenced by at least about 50% increase or decrease (preferably decrease) in the rate of loss of enzyme activity when exposed to various oxidizing conditions. Such oxidizing conditions are exposure to the organic
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oxidant diperdodecanoic acid (DPDA) under the conditions described in the examples.

5 Alkaline stability is measured either by known procedures or by the methods described herein. A substantial change in alkaline stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half life of the enzymatic activity of a mutant when compared to the precursor carbonyl hydrolase. In the case of
10 subtilisins, alkaline stability was measured as a function of autoproteolytic degradation of subtilisin at alkaline pH, e.g. for example, 0.1M sodium phosphate, pH 12 at 25° or 30°C.

15 Thermal stability is measured either by known procedures or by the methods described herein. A substantial change in thermal stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half-life of the catalytic activity of a mutant when exposed to a relatively high temperature and neutral pH as compared to the precursor carbonyl hydrolase. In the case of
20 subtilisins, thermal stability is measured by the autoproteolytic degradation of subtilisin at elevated temperatures and neutral pH, e.g., for example 2mM calcium chloride, 50mM MOPS pH 7.0 at 59°C.

25 The inventors have produced mutant subtilisins containing the substitution of the amino acid residues of B. amyloliquefaciens subtilisin shown in Table I. The wild type amino acid sequence and DNA sequence of B. amyloliquefaciens subtilisin is shown in Fig. 1.
30

TABLE I

	Residue	Replacement Amino Acid
	Tyr21	F A
	Thr22	C
5	Ser24	C
	Asp32	Q S
	Ser33	A T
	Asp36	A G
	Gly46	V
10	Ala48	E V R
	Ser49	C L
	Met50	C F V
	Asn77	D
	Ser87	C
15	Lys94	C
	Val95	C
	Leu96	D
	Tyr104	A C D E F G H I K L M N P Q R S T V W
	Ile107	V
20	Gly110	C R
	Met124	I L
	Asn155	A D H Q T
	Glu156	Q S
	Gly166	C E I L M P S T W Y
25	Gly169	C D E F H I K L M N P Q R T V W Y
	Lys170	E R
	Tyr171	F
	Pro172	E Q
	Phe189	A C D E G H I K L M N P Q R S T V W Y
30	Asp197	R A
	Met199	I
	Ser204	C R L P
	Lys213	R T
	Tyr217	A C D E F G H I K L M N P Q R S T V W
35	Ser221	A C

The different amino acids substituted are represented in Table I by the following single letter designations:

	<u>Amino acid or residue thereof</u>	<u>3-letter symbol</u>	<u>1-letter symbol</u>
5	Alanine	Ala	A
	Glutamate	Glu	E
	Glutamine	Gln	Q
10	Aspartate	Asp	D
	Asparagine	Asn	N
	Leucine	Leu	L
	Glycine	Gly	G
	Lysine	Lys	K
15	Serine	Ser	S
	Valine	Val	V
	Arginine	Arg	R
	Threonine	Thr	T
	Proline	Pro	P
20	Isoleucine	Ile	I
	Methionine	Met	M
	Phenylalanine	Phe	F
	Tyrosine	Tyr	Y
	Cysteine	Cys	C
25	Tryptophan	Trp	W
	Histidine	His	H

Except where otherwise indicated by context, wild-type amino acids are represented by the above three-letter symbols and replaced amino acids by the above single-letter symbols. Thus, if the methionine at residue 50 in *B. amyloliquefaciens* subtilisin is

replaced by phenylalanine, this mutation (mutant) may be designated Met50F or F50. Similar designations are used for multiple mutants.

5 In addition to the amino acids used to replace the residues disclosed in Table I, other replacements of amino acids at these residues are expected to produce mutant subtilisins having useful properties. These residues and replacement amino acids are shown in Table II.

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TABLE II

Residue	Replacement Amino Acid(s)
Tyr-21	L
Thr22	K
Ser24	A
Asp32	
Ser33	G
Gly46	
Ala48	
Ser49	
Met50	L K I V
Asn77	D
Ser87	N
Lys94	R Q
Val95	L I
Tyr104	
Met124	K A
Ala152	C L I T M
Asn155	
Glu156	A T M L Y
Gly166	
Gly169	
Tyr171	K R E Q
Pro172	D N
Phe189	
Tyr217	
Ser221	
Met222	

Each of the mutant subtilisins in Table I contain the replacement of a single residue of the *B. amyloliquefaciens* amino acid sequence. These particular residues were chosen to probe the influence

of such substitutions on various properties of B. amyloliquefacien subtilisin.

Thus, the inventors have identified Met124 and Met222 as important residues which if substituted with another amino acid produce a mutant subtilisin with enhanced oxidative stability. For Met124, Leu and Ile are preferred replacement amino acids. Preferred amino acids for replacement of Met222 are disclosed in EPO Publication No. 0130756.

Various other specific residues have also been identified as being important with regard to substrate specificity. These residues include Tyr104, Ala152, Glu156, Gly166, Gly169, Phe189 and Tyr217 for which mutants containing the various replacement amino acids presented in Table I have already been made, as well as other residues presented below for which mutants have yet to be made.

The identification of these residues, including those yet to be mutated, is based on the inventors' high resolution crystal structure of B. amyloliquefaciens subtilisin to 1.8 Å (see Table III), their experience with in vitro mutagenesis of subtilisin and the literature on subtilisin. This work and the x-ray crystal structures of subtilisin containing covalently bound peptide inhibitors (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449), product complexes (Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303), and transition state analogs (Matthews, D.A., et al. (1975) J. Biol. Chem. 250, 7120-7126; Poulos, T.L., et al. (1976) J. Biol. Chem. 251, 1097-1103), has helped in identifying an extended peptide binding cleft in subtilisin. This substrate binding cleft together with substrate is schematically

diagrammed in Fig. 2, according to the nomenclature
of Schechter, I., et al. (1967) Biochem Bio. Res.
Commun. 27, 157. The scissile bond in the substrate
is identified by an arrow. The P and P' designations
refer to the amino acids which are positioned
respectively toward the amino or carboxy terminus
relative to the scissile bond. The S and S'
designations refer to subsites in the substrate
binding cleft of subtilisin which interact with the
corresponding substrate amino acid residues.

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Atomic Coordinates for the
Apoenzyme Form of *B. Amyloliquefaciens*
Subtilisin to 1.8Å Resolution

1	ALA N	19.434	53.195	-21.754	1	ALA CA	19.811	51.774	-21.965
1	ALA C	18.731	58.995	-21.324	1	ALA O	18.374	51.197	-28.175
1	ALA CB	21.099	51.518	-21.183	2	GLN N	18.268	49.886	-22.841
2	GLN CA	17.219	49.008	-21.434	2	GLN C	17.875	47.786	-28.992
2	GLN O	18.765	47.165	-21.491	2	GLN CB	16.125	48.760	-22.449
2	GLN CG	15.328	47.905	-21.927	2	GLN CD	13.912	47.742	-22.930
2	GLN DE1	13.023	48.612	-22.867	3	SER CA	14.115	46.917	-23.924
3	SER N	17.477	47.205	-19.852	3	SER O	17.950	45.848	-19.437
3	SER C	16.735	44.918	-19.490	3	SER CG	15.590	45.352	-19.229
3	SER CB	18.588	45.838	-18.849	4	VAL CA	17.682	46.210	-17.049
4	VAL N	16.991	43.444	-19.725	4	VAL O	15.946	42.419	-19.639
4	VAL C	16.129	41.934	-18.290	4	VAL CB	17.123	41.178	-18.086
4	VAL CB	16.008	41.622	-20.822	5	PRO N	14.874	48.572	-20.741
4	VAL CG2	16.037	42.266	-22.186	5	PRO C	15.230	42.104	-17.331
5	PRO CA	15.384	41.415	-16.027	5	PRO CB	15.501	39.905	-16.249
5	PRO O	14.885	39.243	-17.146	5	PRO CD	14.150	41.880	-15.243
5	PRO CG	13.841	43.215	-15.921	6	TYR CA	14.044	42.986	-17.417
6	TYR N	16.363	39.240	-15.487	6	TYR O	16.628	37.803	-15.715
6	TYR C	15.359	36.975	-15.528	6	TYR CG	15.224	35.943	-16.235
6	TYR CB	17.824	37.323	-14.834	6	TYR CD2	18.021	35.847	-15.055
6	TYR CD1	18.437	35.452	-16.346	6	TYR CE2	17.496	34.908	-14.071
6	TYR CE1	18.535	34.070	-16.653	6	TYR OH	17.815	33.539	-14.379
6	TYR CZ	18.222	33.154	-15.628	7	GLY CA	18.312	31.838	-15.996
7	GLY N	14.464	37.362	-14.630	7	GLY O	13.211	34.640	-14.374
7	GLY C	12.400	36.535	-15.670	8	VAL CA	11.747	35.478	-15.883
8	VAL N	12.441	37.529	-16.541	8	VAL O	11.777	37.523	-17.836
8	VAL C	12.363	36.433	-18.735	8	VAL CB	11.639	35.716	-19.470
8	VAL CB	11.765	38.900	-18.567	9	SER N	11.106	38.893	-19.943
8	VAL CG2	10.991	39.919	-17.733	9	SER C	13.661	36.318	-18.775
9	SER CA	14.419	35.342	-19.562	9	SER CB	14.188	33.920	-18.945
9	SER O	14.112	33.814	-19.901	10	GLN N	15.926	35.632	-19.505
9	SER CG	16.162	36.747	-20.358	10	GLN C	14.115	33.887	-17.462
10	GLN CA	13.964	32.636	-16.876	10	GLN CB	12.687	31.887	-17.277
10	GLN O	12.785	30.642	-17.413	10	GLN CD	14.125	32.885	-15.410
10	GLN CG	14.295	31.617	-14.588	10	GLN NE2	14.486	31.911	-13.147
10	GLN DE1	14.554	33.068	-12.764	11	ILE CA	14.552	30.960	-12.251
11	ILE N	11.425	32.575	-17.670	11	ILE O	10.373	31.984	-18.182
11	ILE C	10.209	31.792	-19.605	11	ILE CG1	9.173	31.333	-20.180
11	ILE CB	9.132	32.669	-17.475	11	ILE CD1	9.066	34.117	-18.049
11	ILE CG2	9.162	32.655	-15.941	12	LVS CA	7.588	34.648	-17.923
12	LVS N	11.272	32.185	-20.277	12	LVS O	11.388	32.119	-21.722
12	LVS C	10.456	33.086	-22.522	12	LVS CG	10.178	32.703	-23.686
12	LVS CB	11.257	30.444	-22.216	12	LVS CE	12.283	29.830	-21.423
12	LVS CD	12.543	28.517	-22.159	13	ALA N	13.023	27.467	-21.166
12	LVS NZ	14.476	27.680	-20.935	13	ALA C	10.109	34.138	-21.991
13	ALA CA	9.325	35.198	-22.631	13	ALA CB	10.026	35.716	-23.863
13	ALA O	9.338	35.804	-24.901	14	PRO CA	8.885	36.195	-21.565
14	PRO N	11.332	35.950	-23.893	14	PRO O	11.985	36.430	-25.120
14	PRO C	11.786	35.557	-26.317	14	PRO CG	11.778	36.047	-27.645
14	PRO CB	13.462	36.588	-24.692	15	ALA N	13.328	36.978	-23.221
14	PRO CD	12.281	35.936	-22.758	15	ALA C	11.560	34.236	-26.129
15	ALA CA	11.379	33.458	-27.367	15	ALA CB	10.082	33.795	-28.032
15	ALA O	10.088	33.718	-29.278	16	LEU CA	11.552	31.969	-27.062
16	LEU N	9.085	34.138	-27.240	16	LEU O	7.791	34.958	-27.828
16	LEU C	7.912	35.925	-28.521	16	LEU CG	7.362	36.126	-29.588
16	LEU CB	6.746	34.623	-26.698	16	LEU CD2	9.790	33.665	-26.522
16	LEU CD1	8.881	33.234	-27.809	17	MIS CA	6.694	32.287	-26.283
17	MIS N	8.665	36.828	-27.922	17	MIS O	8.898	38.151	-28.538
17	MIS C	9.518	37.981	-29.898	17	MIS CG	9.107	38.622	-30.856
17	MIS CB	9.788	39.188	-27.652	17	MIS CD2	9.185	39.288	-29.262
17	MIS ND1	9.938	39.887	-25.272	17	MIS NE2	8.088	38.924	-25.694
17	MIS CE1	9.224	39.914	-24.144	18	SER CA	8.079	39.328	-24.381
18	SER N	18.443	37.833	-38.822			11.109	36.739	-31.322

18	SEN C	10.139	36.123	-31.343	18	SEN D	10.947	36.112	-31.536
18	SEN CB	12.311	35.799	-31.172	18	SEN DC	13.321	36.480	-30.399
19	GLN N	9.880	35.693	-31.943	19	GLN CA	8.882	36.962	-31.878
19	GLN C	7.142	36.111	-33.303	19	GLN D	6.297	35.972	-34.219
19	GLN CB	7.221	33.849	-32.280	19	GLN CC	7.978	32.882	-31.823
19	GLN CD	6.823	31.707	-31.181	19	GLN CCB	8.719	31.833	-31.466
19	GLN CCB	7.362	30.832	-30.236	20	GLV N	7.285	37.223	-32.587
20	GLV CA	6.369	30.387	-32.859	20	GLV C	5.181	38.492	-31.880
20	GLV D	4.263	39.276	-32.313	21	TYR N	8.202	37.881	-30.761
21	TYR CA	4.118	37.831	-29.763	21	TYR C	4.879	38.832	-28.823
21	TYR D	5.422	38.074	-27.756	21	TYR CB	2.498	36.631	-29.443
21	TYR CC	2.973	31.784	-30.788	21	TYR CCB	1.795	36.332	-31.238
21	TYR CCB	3.690	36.794	-31.397	21	TYR CCB	1.306	33.797	-32.446
21	TYR CCB	3.193	34.261	-32.888	21	TYR CCB	2.083	34.755	-33.847
21	TYR CCB	1.801	36.241	-34.250	22	TYR N	3.902	39.680	-28.288
22	TYR CA	4.262	40.827	-27.129	22	TYR C	3.091	40.922	-26.244
22	TYR D	3.287	41.723	-25.323	22	TYR CB	5.133	41.759	-27.611
22	TYR DCB	4.319	42.437	-21.397	22	TYR CCB	6.476	41.323	-28.229
23	GLV N	1.939	40.285	-26.453	23	GLV CA	8.889	40.000	-23.942
23	GLV C	-0.157	41.631	-26.118	23	GLV D	-1.813	42.895	-28.330
24	SEN N	-0.823	41.967	-27.371	24	SEN CA	-8.897	42.917	-28.812
24	SEN C	-2.383	42.626	-27.864	24	SEN D	-2.813	41.508	-28.168
24	SEN CB	-0.734	43.120	-29.320	24	SEN DC	0.363	43.632	-29.728
25	ASN N	-3.059	43.692	-27.313	25	ASN CA	-4.519	43.887	-27.393
25	ASN C	-9.019	42.873	-26.205	25	ASN D	-6.233	42.668	-26.190
25	ASN CB	-5.165	43.227	-28.700	25	ASN CC	-6.960	44.170	-29.883
25	ASN CCB	-4.965	43.767	-31.083	25	ASN CCB	-4.747	45.461	-29.594
26	VAL N	-4.177	42.649	-25.292	26	VAL CA	-4.674	41.679	-24.143
26	VAL C	-4.792	41.852	-22.987	26	VAL D	-3.838	43.419	-27.689
26	VAL CB	-3.714	40.903	-23.821	26	VAL CCB	-4.160	39.802	-22.948
26	VAL CCB	-3.988	39.576	-25.018	27	LVS N	-5.910	42.613	-21.301
27	LVS CA	-6.133	43.526	-21.175	27	LVS C	-5.815	42.872	-19.841
27	LVS D	-6.605	41.973	-19.413	27	LVS CB	-7.590	43.981	-21.149
27	LVS CC	-8.046	44.575	-22.490	27	LVS CD	-9.321	45.302	-22.820
27	LVS CCB	-10.304	45.497	-23.137	27	LVS CCB	-9.686	46.253	-24.264
28	VAL N	-4.818	43.462	-19.200	28	VAL CA	-4.457	42.930	-17.997
28	VAL C	-4.758	43.959	-18.828	28	VAL D	-4.209	45.893	-16.817
28	VAL CB	-2.926	42.666	-17.882	28	VAL CCB	-2.466	42.103	-16.389
28	VAL CCB	-2.667	41.805	-19.173	29	ALA N	-5.484	43.527	-18.813
29	ALA CA	-5.747	44.330	-14.639	29	ALA C	-4.750	44.010	-13.553
29	ALA D	-4.666	42.843	-13.104	29	ALA CB	-7.172	46.187	-16.181
30	VAL N	-4.957	45.033	-13.072	30	VAL CA	-3.166	46.962	-11.910
30	VAL C	-3.958	45.409	-10.681	30	VAL D	-4.195	46.648	-10.878
30	VAL CB	-1.886	45.810	-12.149	30	VAL CCB	-0.906	45.981	-10.998
30	VAL CCB	-1.853	45.236	-13.307	31	ILE N	-4.814	44.918	-9.877
31	ILE CA	-5.328	44.846	-8.679	31	ILE C	-4.346	44.933	-7.946
31	ILE D	-3.825	43.913	-6.997	31	ILE CB	-6.457	43.774	-8.901
31	ILE CCB	-7.298	43.707	-9.798	31	ILE CCB	-7.278	46.838	-7.225
31	ILE CCB	-8.617	42.856	-9.717	32	ASP N	-6.044	46.193	-7.227
32	ASP CA	-2.944	46.467	-6.235	32	ASP C	-3.071	47.889	-3.705
32	ASP D	-4.197	48.418	-5.392	32	ASP CB	-1.695	46.129	-7.092
32	ASP CC	-0.483	48.782	-6.273	32	ASP CCB	0.834	46.592	-6.576
32	ASP CCB	-0.881	46.429	-5.330	33	SEN N	-1.931	48.912	-3.394
33	SEN CA	-1.895	49.837	-4.801	33	SEN C	-3.982	48.976	-3.803
33	SEN D	-1.706	52.126	-5.363	33	SEN CB	-0.621	49.922	-3.939
33	SEN DC	0.333	50.028	-4.774	34	GLV N	-2.173	48.749	-7.884
34	GLV CA	-2.255	51.728	-8.165	34	GLV C	-1.833	51.648	-9.057
34	GLV D	-0.144	50.831	-8.761	35	ILE N	-0.963	52.431	-10.102
35	ILE CA	0.208	52.438	-10.995	35	ILE C	0.568	53.919	-11.263
35	ILE D	-0.327	54.638	-11.764	35	ILE CB	-0.842	51.604	-12.367
35	ILE CCB	-0.530	50.210	-12.097	35	ILE CCB	1.149	51.761	-13.362
35	ILE CCB	-0.962	49.488	-13.424	36	ASP N	1.816	54.283	-10.971
36	ASP CA	2.339	55.618	-11.232	36	ASP C	2.281	55.956	-12.782

34	ASP D	3.804	55.471	-13.579
34	ASP CG	4.339	57.099	-10.804
34	ASP BD2	3.448	57.277	-10.263
37	SER CA	2.183	57.221	-14.512
37	SER O	2.545	58.303	-16.151
37	SER DG	-8.010	59.133	-13.879
38	SER CA	4.261	59.505	-14.487
38	SER D	6.543	59.251	-15.285
38	SER DG	5.376	59.865	-12.234
39	MIS CA	6.437	56.574	-15.291
39	MIS D	5.738	55.078	-17.419
39	MIS CG	8.014	56.409	-14.456
39	MIS CD2	8.769	56.345	-13.389
39	MIS ME2	9.984	53.910	-13.808
40	PRD CA	7.988	56.697	-18.831
40	PRD O	8.832	55.097	-20.578
40	PRD CG	10.053	57.405	-17.982
41	ASP M	8.481	54.328	-18.485
41	ASP OD1	10.325	51.395	-20.429
41	ASP CB	9.799	52.239	-18.224
41	ASP C	7.311	52.163	-18.839
42	LEU M	6.185	52.803	-10.558
42	LEU C	3.924	52.907	-19.376
42	LEU CB	4.421	52.158	-17.808
42	LEU CD1	4.535	51.546	-14.581
43	LVS M	3.018	52.135	-19.946
43	LVS C	0.637	52.156	-20.018
43	LVS CB	2.021	52.389	-22.169
43	LVS CD	8.998	52.862	-24.339
43	LVS M2	8.337	51.757	-26.418
44	VAL CA	-1.487	52.639	-18.765
44	VAL O	-2.623	53.906	-20.434
44	VAL CG1	-2.724	52.941	-16.582
45	ALA M	-3.494	51.951	-19.871
45	ALA C	-5.841	52.507	-20.053
45	ALA CB	-4.831	50.580	-21.389
46	GLY CA	-7.082	52.837	-13.081
46	GLY O	-5.938	52.806	-16.035
47	GLY CA	-8.014	52.246	-14.388
47	GLY D	-9.988	53.481	-14.185
48	ALA CA	-10.255	52.870	-11.382
48	ALA O	-9.066	51.720	-9.725
49	SER M	-10.149	53.547	-9.837
49	SER C	-10.047	52.984	-6.783
49	SER CB	-9.092	54.588	-7.029
50	MET M	-10.835	52.087	-5.932
50	MET C	-11.463	51.962	-3.561
50	MET CB	-12.012	50.818	-4.996
50	MET SD	-13.466	49.889	-7.256
51	VAL M	-10.427	52.768	-3.422
51	VAL C	-10.630	54.562	-1.987
51	VAL CB	-8.443	53.155	-2.008
51	VAL CG2	-7.764	53.815	-2.302
52	PRD CA	-12.372	53.933	-0.821
52	PRD O	-11.771	50.228	-0.925
52	PRD CG	-13.583	54.183	0.085
53	SER M	-10.442	56.906	0.299
53	SER C	-8.420	58.245	-0.324
53	SER CB	-9.004	57.787	2.069
54	GLU M	-8.254	57.523	-1.393
54	GLU C	-7.767	57.383	-2.785
54	GLU CB	-6.134	56.599	-2.154
54	GLU CD	-4.044	54.049	-0.098

36	ASP CB	3.732	55.728	-10.514
36	ASP OD1	3.755	57.974	-11.629
37	SER M	1.304	56.822	-13.111
37	SER C	2.377	58.895	-14.949
37	SER CB	-0.093	58.049	-14.788
38	SER M	3.163	58.614	-14.081
38	SER C	5.444	58.705	-14.992
38	SER CB	4.742	60.435	-13.398
39	MIS M	5.454	57.390	-14.892
39	MIS C	6.681	56.401	-16.778
39	MIS CB	6.437	55.203	-14.515
39	MIS MD1	8.795	54.356	-15.561
39	MIS CE1	9.970	53.930	-15.130
40	PRD M	7.087	56.836	-17.387
40	PRD C	8.154	55.280	-19.357
40	PRD CB	9.247	57.533	-19.161
40	PRD CD	8.988	57.452	-16.776
41	ASP OD2	11.148	50.399	-18.668
41	ASP CG	10.473	51.387	-19.211
41	ASP CA	8.645	52.959	-18.966
41	ASP D	7.396	50.947	-18.977
42	LEU CA	4.892	52.147	-18.466
42	LEU O	3.993	54.163	-19.490
42	LEU CG	5.182	51.363	-15.946
42	LEU CD2	5.273	49.877	-16.358
43	LVS CA	1.893	52.685	-20.721
43	LVS O	0.584	50.920	-19.820
43	LVS CG	0.685	52.436	-22.910
43	LVS CE	-0.180	52.584	-25.260
44	VAL M	-0.191	53.035	-19.490
44	VAL C	-2.571	52.887	-19.731
44	VAL CB	-1.480	53.351	-17.383
44	VAL CG2	-0.197	53.194	-16.553
45	ALA CA	-4.619	51.977	-20.810
45	ALA O	-6.783	53.885	-20.703
46	GLY M	-5.918	52.356	-18.768
46	GLY C	-6.987	52.443	-16.538
47	GLY M	-8.092	52.658	-15.793
47	GLY C	-9.179	52.757	-13.572
48	ALA M	-9.221	52.466	-12.330
48	ALA C	-9.790	52.675	-9.968
48	ALA CB	-11.558	52.100	-11.617
49	SER CA	-9.752	53.355	-7.652
49	SER O	-11.972	53.677	-6.988
49	SER DG	-8.879	54.255	-5.650
50	MET CA	-11.852	51.549	-4.974
50	MET O	-11.997	51.398	-2.575
50	MET CG	-11.912	49.463	-6.389
50	MET CE	-12.808	50.111	-0.983
51	VAL CA	-9.968	53.170	-2.067
51	VAL O	-10.237	55.437	-2.682
51	VAL CG1	-7.892	53.579	-0.631
52	PRD M	-11.621	54.693	-1.056
52	PRD C	-11.498	57.123	-0.440
52	PRD CB	-13.480	55.594	0.264
52	PRD CD	-12.164	53.620	-0.175
53	SER CA	-9.538	57.982	0.682
53	SER O	-7.679	59.224	-0.038
53	SER DG	-8.256	56.521	2.127
54	GLU CA	-7.284	57.648	-2.621
54	GLU O	-7.533	56.243	-4.379
54	GLU CG	-5.289	56.959	-0.927
54	GLU MD1	-5.645	55.406	-1.068

54	ELU DE2	-3.000	55.777	0.271	55	THR H	-0.571	58.251	-4.249
55	THR CA	-9.433	58.121	-5.441	55	THR C	-8.744	58.139	-6.779
55	THR B	-9.433	57.919	-7.810	55	THR CB	-10.986	59.200	-5.383
55	THR DC1	-9.885	60.510	-5.418	55	THR CC2	-11.432	59.143	-4.817
56	ASN H	-7.482	58.403	-6.877	56	ASN WD2	-4.930	61.179	-9.881
56	ASN DD1	-5.875	58.967	-10.377	56	ASN CG	-5.273	59.925	-5.355
56	ASN CB	-5.898	59.494	-8.208	56	ASN CA	-6.742	58.425	-8.200
56	ASN C	-6.012	57.894	-8.305	56	ASN D	-5.184	56.864	-7.470
57	PRD H	-6.342	56.241	-9.258	57	PRD CG	-7.123	55.257	-11.177
57	PRD CD	-7.384	56.433	-10.272	57	PRD CB	-6.644	54.178	-10.235
57	PRD CA	-5.679	54.941	-9.332	57	PRD C	-4.301	55.082	-9.946
57	PRD D	-3.589	54.128	-9.945	58	PME H	-3.998	56.262	-10.491
58	PME CA	-2.747	56.577	-11.222	58	PME C	-1.712	57.129	-10.253
58	PME D	-0.635	57.497	-10.680	58	PME C9	-2.943	57.582	-12.423
58	PME CG	-3.983	56.968	-13.357	58	PME CD1	-3.756	55.788	-14.859
58	PME CD2	-5.211	57.630	-13.459	58	PME CE1	-6.722	55.255	-14.928
58	PME CE2	-6.194	57.895	-14.276	58	PME C2	-5.949	55.939	-15.051
59	GLN H	-2.044	57.119	-8.990	59	GLN CA	-1.172	57.583	-7.934
59	GLN C	-0.887	56.483	-7.800	59	GLN D	-1.639	56.883	-6.115
59	GLN CB	-1.862	58.668	-7.889	59	GLN CC	-8.942	59.261	-6.034
59	GLN CD	-1.790	60.157	-5.150	59	GLN DE1	-1.404	61.288	-4.836
59	GLN ME2	-2.959	59.685	-6.742	60	ASP H	0.410	55.895	-7.211
60	ASP CA	0.851	54.792	-6.304	60	ASP C	1.631	55.267	-5.090
60	ASP D	2.827	55.550	-5.231	60	ASP CB	1.596	53.744	-7.180
60	ASP CG	2.077	52.538	-6.380	60	ASP DD1	1.746	52.357	-5.190
60	ASP DD2	2.915	51.841	-7.830	61	ASN H	0.959	55.265	-3.950
61	ASN WD2	-1.364	57.747	-2.347	61	ASN DD1	0.666	58.566	-2.875
61	ASN CG	-8.048	57.670	-2.399	61	ASN CB	0.531	56.401	-1.784
61	ASN CA	1.557	55.734	-2.700	61	ASN C	2.291	54.432	-1.940
61	ASN D	2.933	54.862	-8.902	62	ASN H	2.210	53.434	-2.468
62	ASN CA	2.877	52.348	-1.709	62	ASN C	4.124	51.893	-2.479
62	ASN D	4.951	51.313	-1.770	62	ASN CB	1.783	51.319	-1.421
62	ASN CG	2.371	50.183	-8.697	62	ASN DD1	2.633	49.877	-1.343
62	ASN WD2	2.422	50.208	0.601	63	SER H	4.152	52.184	-3.741
63	SER CA	5.189	51.696	-4.789	63	SER C	5.871	50.256	-3.209
63	SER D	5.593	49.790	-6.269	63	SER CB	6.523	51.958	-4.812
63	SER DG	6.871	50.698	-3.418	64	MIS H	4.282	49.475	-4.639
64	MIS CA	3.994	48.855	-4.935	64	MIS C	3.366	47.759	-6.261
64	MIS D	3.861	46.974	-7.108	64	MIS CB	3.184	47.581	-3.747
64	MIS CG	3.144	46.821	-3.726	64	MIS DD1	2.187	45.247	-4.241
64	MIS CD2	4.854	45.194	-3.135	64	MIS CE1	2.416	43.966	-4.054
64	MIS ME2	3.556	43.920	-3.368	65	GLY H	2.287	48.428	-6.587
65	GLY CA	1.552	48.264	-7.830	65	GLY C	2.392	48.636	-9.837
65	GLY D	2.238	48.878	-10.134	66	THR H	3.233	49.659	-8.832
66	THR CA	4.064	50.117	-9.954	66	THR C	5.889	49.809	-10.291
66	THR D	5.333	48.789	-11.461	66	THR C9	4.744	51.511	-9.667
66	THR DC1	3.437	52.425	-9.406	66	THR CC2	5.534	52.878	-10.849
67	MIS H	5.685	48.443	-9.274	67	MIS CA	6.783	47.361	-9.458
67	MIS C	6.091	46.141	-10.143	67	MIS D	6.649	45.638	-11.150
67	MIS CB	7.308	47.871	-8.064	67	MIS CG	8.595	46.275	-8.148
67	MIS DD1	8.590	44.907	-8.276	67	MIS CD2	9.904	46.678	-8.874
67	MIS CE1	9.857	44.491	-8.299	67	MIS ME2	10.678	45.514	-8.186
68	VAL H	4.892	45.749	-9.731	68	VAL CA	4.142	46.687	-10.266
68	VAL C	3.856	44.868	-11.740	68	VAL D	4.114	43.942	-12.535
68	VAL CB	2.939	44.252	-9.386	68	VAL CC1	1.988	43.268	-10.820
68	VAL CC2	3.319	43.785	-8.888	69	ALA H	3.373	46.849	-12.113
69	ALA CA	3.037	46.668	-13.429	69	ALA C	4.193	46.390	-14.411
69	ALA D	4.028	45.913	-13.565	69	ALA C9	2.332	47.851	-13.386
70	GLY H	5.348	46.782	-13.914	70	GLY CA	6.595	46.885	-14.670
70	GLY C	7.846	45.378	-13.821	70	GLY D	7.684	45.154	-16.119
71	THR H	6.820	44.431	-14.138	71	THR CA	7.177	43.819	-14.446
71	THR C	6.224	42.586	-13.543	71	THR D	6.682	41.828	-16.495
71	THR CB	7.119	42.870	-13.191	71	THR DC1	8.191	42.592	-12.390

71	YHR CG2	7.274	48.583	-13.596
72	VAL CA	3.976	42.491	-16.494
72	VAL B	6.341	42.380	-18.860
72	VAL CG1	1.512	42.488	-17.178
73	ALA M	4.504	44.417	-17.988
73	ALA C	5.433	44.333	-19.355
73	ALA CB	3.107	45.441	-19.433
74	ALA CA	7.478	47.591	-18.959
74	ALA B	7.959	46.640	-21.054
75	LEU M	7.658	48.784	-21.839
75	LEU C	9.192	48.568	-22.966
75	LEU CB	7.548	50.471	-22.809
75	LEU CD1	6.079	52.436	-22.300
76	ASN M	9.147	48.103	-24.169
76	ASN DD1	10.950	45.840	-27.928
76	ASN CA	10.810	46.651	-25.988
76	ASN C	10.783	49.048	-25.443
77	ASN M	11.804	49.464	-25.071
77	ASN C	13.707	51.029	-25.368
77	ASN CB	11.335	52.076	-25.117
77	ASN DD1	12.032	51.366	-22.917
78	SER M	14.125	52.267	-25.164
78	SER C	15.810	52.742	-23.436
78	SER CB	15.905	53.941	-25.587
79	ILE M	14.058	52.565	-22.529
79	ILE C	14.617	51.683	-20.230
79	ILE CB	14.671	54.174	-20.497
79	ILE CG2	14.997	55.320	-21.612
80	GLY M	14.995	51.768	-18.981
80	GLY C	14.612	49.448	-18.219
81	VAL M	13.513	48.766	-17.980
81	VAL C	12.511	46.919	-19.217
81	VAL CB	13.001	46.755	-16.677
81	VAL CG2	11.438	47.261	-16.231
82	LEU CA	11.312	45.020	-20.256
82	LEU B	10.858	43.356	-18.600
82	LEU CG	11.430	43.568	-22.366
82	LEU CD2	12.359	42.675	-23.192
83	GLY CA	8.133	43.321	-19.114
83	GLY B	8.546	41.822	-21.026
84	VAL CA	6.973	39.807	-19.888
84	VAL D	6.424	39.472	-22.194
84	VAL CG1	5.680	37.677	-19.557
85	ALA M	5.156	40.926	-21.024
85	ALA C	4.213	42.483	-22.396
85	ALA CB	2.846	40.443	-21.748
86	PRO CA	5.413	46.635	-23.285
86	PRO B	4.291	46.605	-23.849
86	PRO CG	7.030	43.466	-24.546
87	SER M	3.548	44.676	-24.769
87	SER C	1.103	45.132	-24.897
87	SER CB	2.401	44.777	-26.927
88	ALA M	1.017	44.564	-23.742
88	ALA CA	-0.273	44.353	-23.084
88	ALA B	-0.174	46.717	-22.435
89	SER CG	-4.146	47.102	-24.280
89	SER CA	-3.801	46.867	-22.227
89	SER B	-3.793	45.864	-20.209
90	LEU CA	-2.378	47.667	-18.593
90	LEU B	-3.582	49.604	-18.215
90	LEU CG	-0.233	47.851	-17.176
90	LEU CD2	1.160	49.524	-17.067
91	YHR CA	-5.258	48.678	-16.137

72	VAL M	6.938	42.887	-13.427
72	VAL C	6.312	43.084	-17.831
72	VAL CB	2.514	42.867	-16.885
77	VAL CG2	2.142	42.327	-14.723
73	ALA CA	4.587	45.091	-19.167
73	ALA B	5.062	47.188	-20.216
74	ALA M	6.544	46.429	-18.435
74	ALA C	7.740	47.648	-20.342
74	ALA CB	8.453	47.446	-17.925
75	LEU CA	7.812	48.968	-22.456
75	LEU B	10.162	48.750	-22.253
75	LEU CG	6.123	50.913	-22.379
75	LEU CD2	5.896	50.462	-23.405
76	ASN DD2	12.385	46.432	-26.384
76	ASN CG	11.195	46.274	-26.862
76	ASN CA	10.359	47.738	-24.938
76	ASN B	10.157	49.479	-26.419
77	ASN CA	12.220	50.957	-25.481
77	ASN D	14.364	49.979	-25.323
77	ASN CG	11.250	52.027	-23.616
77	ASN DD2	10.294	52.741	-23.025
78	SER CA	15.513	52.614	-24.906
78	SER B	16.982	53.871	-23.164
78	SER CG	15.926	53.870	-26.999
79	ILE CA	15.155	52.784	-21.120
79	ILE B	13.843	50.841	-20.679
79	ILE CG1	12.945	54.032	-20.814
79	ILE CD1	12.135	55.176	-20.155
80	GLY CA	14.474	50.940	-17.913
80	GLY B	15.719	48.994	-18.544
81	VAL CA	13.411	47.286	-18.061
81	VAL B	12.260	47.739	-20.117
81	VAL CG1	14.030	47.084	-15.573
82	LEU M	12.126	45.645	-19.216
82	LEU C	10.390	44.028	-19.510
82	LEU CB	12.206	44.219	-21.229
82	LEU CD1	10.796	44.657	-23.223
83	GLY M	9.131	44.180	-19.816
83	GLY C	8.027	42.011	-19.925
84	VAL M	7.272	41.112	-19.283
84	VAL C	6.164	40.830	-21.140
84	VAL CB	6.256	38.920	-18.841
84	VAL CG2	7.190	38.507	-17.705
85	ALA CA	4.217	41.194	-22.158
85	ALA B	3.260	43.401	-22.038
86	PRO M	5.240	43.186	-23.059
86	PRO C	4.321	45.371	-23.947
86	PRO CB	6.822	44.784	-23.813
86	PRO CD	6.977	42.440	-23.636
87	SER CA	2.489	45.324	-25.529
87	SER B	8.162	45.513	-25.619
87	SER CG	3.591	45.143	-27.583
88	ALA CB	-0.163	43.510	-21.820
88	ALA C	-0.098	45.717	-22.690
89	SER M	-2.219	45.691	-22.678
89	SER CB	-6.343	46.903	-22.898
89	SER C	-3.136	46.780	-20.727
90	LEU M	-2.446	47.656	-20.937
90	LEU C	-3.483	48.438	-17.864
90	LEU CB	-0.951	48.273	-18.426
90	LEU CD1	-0.026	46.361	-17.219
91	YHR M	-6.264	47.966	-16.938
92	YHR C	-4.873	48.758	-14.685

91 TYR D	-4.494	47.749	-14.023
91 TYR CG	-7.894	48.237	-17.741
91 TYR CD2	-7.971	49.275	-18.149
91 TYR CE2	-8.315	49.421	-19.492
91 TYR DM	-8.102	48.752	-21.764
92 ALA CA	-4.549	50.199	-12.707
92 ALA D	-4.723	50.898	-12.050
93 VAL M	-5.959	48.993	-31.129
93 VAL C	-6.708	49.014	-8.899
93 VAL CB	-7.957	47.555	-10.611
93 VAL CG2	-8.195	47.370	-12.872
94 LYS CA	-6.378	50.464	-6.999
94 LYS D	-8.458	50.480	-5.783
94 LYS CG	-5.394	52.320	-5.467
94 LYS CE	-4.399	54.208	-4.199
95 VAL M	-6.909	49.071	-5.026
95 VAL C	-6.919	48.499	-2.568
95 VAL CB	-8.104	47.030	-4.319
95 VAL CG2	-4.900	46.100	-4.332
96 LEU CA	-4.782	49.103	-1.486
96 LEU D	-3.942	51.121	-2.336
96 LEU CG	-3.593	46.799	-2.072
96 LEU CD2	-4.489	46.082	-1.045
97 GLY CA	-3.890	52.307	0.287
97 GLY D	-1.619	51.463	0.165
98 ALA CB	-0.428	55.470	1.510
98 ALA C	0.108	53.118	1.917
99 ASP M	-0.504	52.573	2.912
99 ASP DD1	-2.730	50.902	4.003
99 ASP CB	-0.648	51.603	5.175
99 ASP C	0.146	50.165	3.320
100 GLY M	-0.424	49.883	2.148
100 GLY C	-2.520	47.651	2.002
101 SER M	-2.342	48.128	2.908
101 SER C	-4.750	47.894	2.532
101 SER CB	-3.716	47.447	4.817
102 GLY M	-5.821	47.092	2.577
102 GLY C	-8.164	46.536	2.528
103 GLN M	-9.377	47.058	2.498
103 GLN C	-10.963	45.232	2.022
103 GLN CB	-11.671	47.307	3.274
103 GLN CD	-12.360	49.104	4.915
103 GLN ME2	-13.419	49.197	6.112
104 TYR CA	-12.068	43.124	1.508
104 TYR D	-12.939	43.276	-0.687
104 TYR CG	-11.629	40.829	2.472
104 TYR CD2	-10.379	40.959	1.860
104 TYR CE2	-9.352	40.057	2.171
104 TYR DM	-8.481	38.191	3.324
105 SER CA	-14.077	45.166	-0.034
105 SER D	-14.759	45.935	-2.250
105 SER CG	-15.289	47.039	1.450
106 TRP CA	-12.421	47.391	-1.948
106 TRP D	-12.821	46.648	-4.245
106 TRP CG	-11.845	49.111	-8.206
106 TRP CD2	-10.658	49.812	0.591
106 TRP CE2	-11.359	50.573	1.561
106 TRP CZ2	-10.671	51.318	2.500
106 TRP CM2	-9.293	51.291	2.455
107 ILE CA	-10.765	44.250	-3.325
107 ILE D	-11.695	43.474	-5.398
107 ILE CG1	-8.634	43.784	-1.934
107 ILE CD1	-8.243	42.998	-0.627

91 TYR CB	-6.684	48.093	-16.314
91 TYR CD1	-6.595	47.415	-18.755
91 TYR CE1	-6.985	47.372	-20.098
91 TYR CZ	-7.794	48.502	-20.463
92 ALA M	-4.895	49.958	-14.104
92 ALA C	-5.823	50.033	-11.903
92 ALA CB	-3.997	51.621	-12.488
93 VAL CA	-7.183	48.854	-18.325
93 VAL D	-6.181	47.993	-8.372
93 VAL CG1	-9.213	47.488	-9.725
94 LYS M	-6.907	50.217	-8.327
94 LYS C	-7.331	49.985	-5.894
94 LYS CB	-6.051	51.974	-4.818
94 LYS CD	-4.868	53.785	-5.582
94 LYS NZ	-3.735	55.544	-4.387
95 VAL CA	-7.646	48.457	-3.920
95 VAL D	-7.425	48.156	-1.501
95 VAL CG1	-8.868	46.852	-5.619
96 LEU M	-5.676	48.974	-2.604
96 LEU C	-4.331	50.559	-1.321
96 LEU CB	-3.509	48.241	-1.573
96 LEU CD1	-2.207	46.184	-2.163
97 GLY M	-4.324	50.975	-0.086
97 GLY C	-2.363	52.437	0.385
98 ALA M	-1.954	53.648	0.758
98 ALA CA	-0.563	54.068	0.965
98 ALA D	1.393	52.921	1.663
99 ASP DD2	-2.631	51.042	6.151
99 ASP CG	-2.083	51.131	5.040
99 ASP CA	0.101	51.610	3.055
99 ASP D	0.735	49.313	4.029
100 GLY CA	-8.343	48.521	1.615
100 GLY D	-1.649	46.512	1.479
101 SER CA	-3.542	47.308	3.315
101 SER D	-4.758	48.972	1.907
101 SER CG	-4.411	48.434	5.209
102 GLY CA	-7.877	47.422	1.894
102 GLY D	-7.888	45.431	3.030
103 GLN CA	-10.535	46.297	3.020
103 GLN C	-10.779	45.482	0.817
103 GLN CG	-11.368	48.005	4.586
103 GLN DE1	-12.159	49.816	5.902
104 TYR M	-11.611	44.141	2.451
104 TYR C	-13.031	43.690	0.473
104 TYR CB	-12.697	41.864	2.143
104 TYR CD1	-11.819	39.789	3.377
104 TYR CE1	-10.809	38.085	3.707
104 TYR CZ	-9.564	39.022	3.001
105 SER M	-13.509	46.572	0.903
105 SER C	-14.172	45.970	-1.159
105 SER CB	-15.080	46.121	0.601
106 TRP M	-15.079	46.625	-0.834
106 TRP C	-11.895	46.436	-3.012
106 TRP CB	-11.321	48.254	-1.355
106 TRP CD1	-12.062	49.524	0.264
106 TRP ME1	-12.691	50.358	1.340
106 TRP CE3	-9.275	49.852	0.576
106 TRP CZ3	-8.568	50.563	1.525
107 ILE M	-11.339	45.330	-2.481
107 ILE C	-11.955	43.594	-4.198
107 ILE CB	-9.946	43.183	-2.523
107 ILE CG2	-9.632	41.930	-3.381
108 ILE M	-12.094	43.292	-3.577

100	IIE CA	-14.314	42.722	-4.321
100	IIE D	-14.894	43.328	-6.552
100	IIE CG1	-14.726	41.077	-2.482
100	IIE CD1	-15.452	40.845	-1.131
109	ASN CA	-15.204	46.018	-5.916
109	ASN B	-14.660	46.272	-8.235
109	ASN CG	-16.528	47.400	-4.353
109	ASN MD2	-14.633	48.447	-3.442
110	GLY CA	-11.952	45.917	-7.065
110	GLY D	-11.929	44.929	-10.034
111	IIE CA	-12.603	42.334	-9.099
111	IIE D	-13.921	42.384	-11.148
111	IIE CG1	-13.421	40.501	-7.655
111	IIE CD1	-13.588	39.706	-6.336
112	GLU CA	-16.118	43.376	-10.046
112	GLU D	-16.467	44.130	-12.246
112	GLU CG	-17.047	42.917	-8.135
112	GLU DE1	-19.041	40.866	-8.016
113	TRP M	-15.094	45.403	-10.971
113	TRP C	-14.076	45.663	-13.140
113	TRP CB	-13.882	47.553	-11.434
113	TRP CD1	-14.148	49.736	-12.681
113	TRP ME1	-13.597	50.443	-13.723
113	TRP CE3	-11.451	47.645	-13.809
113	TRP CZ3	-10.610	47.899	-14.879
114	ALA M	-13.089	44.801	-12.832
114	ALA C	-13.199	43.179	-14.752
114	ALA CB	-11.299	43.192	-13.140
115	IIE CA	-15.070	41.640	-14.097
115	IIE D	-16.077	42.225	-17.070
115	IIE CG1	-15.210	39.836	-13.043
115	IIE CD1	-16.004	39.411	-11.743
116	ALA CA	-17.390	44.440	-16.050
116	ALA D	-17.323	45.255	-18.343
117	ASN M	-15.423	45.390	-17.122
117	ASN C	-13.827	44.974	-19.034
117	ASN CB	-13.615	46.958	-17.426
117	ASN DD1	-14.565	49.082	-17.773
118	ASN M	-14.223	43.725	-18.967
118	ASN C	-12.240	42.444	-19.843
118	ASN CB	-14.247	42.863	-21.279
118	ASN DD1	-16.510	42.321	-20.759
119	MET M	-11.606	42.500	-18.675
119	MET C	-10.025	40.734	-18.920
119	MET CB	-9.810	42.461	-17.055
119	MET SD	-8.788	44.943	-17.526
120	ASP M	-8.904	40.437	-19.584
120	ASP C	-7.822	38.390	-18.856
120	ASP CB	-7.555	39.156	-21.236
120	ASP DD1	-7.801	40.706	-23.084
121	VAL M	-7.021	39.117	-18.115
121	VAL C	-6.296	39.534	-15.786
121	VAL CB	-6.755	38.507	-17.496
122	IIE CA	-4.787	37.916	-18.846
122	IIE D	-6.248	39.799	-13.397
122	IIE CG1	-4.829	38.012	-12.469
122	IIE CD1	-8.686	40.392	-13.063
123	ASN CA	-9.976	39.788	-12.393
123	ASN B	-3.165	39.854	-11.232
123	ASN CG	-3.708	41.631	-9.833
123	ASN MD2	-0.692	40.848	-10.777
124	MET CA	-0.366	40.747	-9.720
		-3.650	39.973	-7.430

200	IIE C	-14.439	43.694	-3.386
200	IIE CB	-15.246	42.265	-3.320
200	IIE CG2	-16.568	42.024	-4.093
209	ASN M	-14.751	46.958	-4.981
209	ASN C	-14.232	46.067	-7.884
209	ASN CB	-15.280	47.359	-5.207
209	ASN MD1	-17.455	46.695	-6.446
210	GLY M	-12.951	45.908	-6.774
210	GLY C	-12.108	44.712	-8.812
211	IIE M	-12.379	43.539	-8.246
211	IIE C	-13.859	42.560	-9.942
211	IIE CB	-12.734	40.948	-8.364
212	IIE CG2	-13.122	39.791	-9.347
212	GLU M	-14.893	43.075	-9.280
212	GLU CB	-15.872	44.347	-11.171
212	GLU CD	-17.229	43.899	-9.141
212	GLU DE2	-18.724	41.826	-8.685
213	TRP CA	-19.123	41.928	-9.866
213	TRP D	-14.756	46.400	-12.000
213	TRP CG	-14.319	45.932	-14.332
213	TRP CD2	-13.486	48.556	-12.481
213	TRP CE2	-12.441	48.552	-13.463
213	TRP CZ2	-12.545	49.761	-14.215
213	TRP CM2	-11.696	50.045	-15.274
214	ALA CA	-10.752	49.074	-15.683
214	ALA D	-12.333	44.065	-13.874
215	IIE M	-12.963	43.074	-15.978
215	IIE C	-14.174	42.540	-14.319
215	IIE CB	-15.928	42.485	-15.856
215	IIE CG2	-16.000	40.840	-13.922
216	ALA M	-17.151	40.168	-14.755
216	ALA C	-16.534	43.527	-15.267
216	ALA CB	-16.706	45.069	-17.278
217	ASN CA	-18.011	45.510	-15.151
217	ASN D	-14.553	45.967	-18.139
217	ASN CG	-12.997	45.436	-19.820
217	ASN MD2	-14.400	48.177	-16.939
218	ASN CA	-14.931	48.249	-15.736
218	ASN D	-13.760	42.642	-19.832
218	ASN CG	-11.617	42.309	-20.932
218	ASN MD2	-15.737	43.060	-21.395
219	MET CA	-16.136	44.096	-22.133
219	MET D	-10.232	42.222	-18.478
219	MET CB	-10.888	39.838	-18.759
219	MET CE	-9.880	43.883	-16.582
220	ASP CA	-9.982	46.061	-18.263
220	ASP D	-8.480	39.110	-20.030
220	ASP CG	-8.038	37.109	-18.690
220	ASP DD2	-8.237	39.730	-22.654
221	VAL CA	-9.327	39.135	-22.739
221	VAL D	-6.224	38.601	-16.976
221	VAL CG1	-6.284	40.788	-15.909
222	IIE M	-3.758	38.176	-16.427
222	IIE C	-6.318	38.978	-14.590
222	IIE CB	-5.820	39.262	-12.627
222	IIE CG2	-7.476	39.604	-12.466
223	ASN M	-7.221	39.883	-10.956
223	ASN C	-4.263	40.222	-12.110
223	ASN CB	-3.502	40.406	-9.861
223	ASN DD1	-1.820	40.478	-11.697
224	MET M	-8.063	38.990	-11.010
224	MET C	-3.458	39.604	-8.832
		-2.423	39.603	-6.614

124	NET D	-2.304	30.308	-6.090	124	NET C8	-6.043	39.387	-6.890
124	NET CG	-6.198	40.282	-7.673	124	NET S2	-7.883	39.472	-6.430
124	NET C1	-7.949	38.093	-7.942	129	STR M	-1.454	40.496	-6.502
129	STR CA	-8.193	40.287	-3.769	129	STR C	-8.422	40.712	-6.326
129	STR D	0.239	41.637	-3.803	129	STR C0	1.021	41.027	-6.328
129	STR D0	1.444	40.496	-7.575	126	LEU M	-1.433	40.075	-3.773
126	LEU CA	-1.842	40.347	-2.386	126	LEU C	-2.438	39.816	-1.807
126	LEU D	-2.844	38.136	-2.529	126	LEU C8	-2.791	41.868	-2.410
126	LEU CG	-3.988	41.447	-3.333	126	LEU CD1	-8.378	41.131	-2.379
126	LEU CD2	-6.170	42.760	-6.873	127	GLY M	-2.522	39.082	-9.481
127	GLY CA	-3.835	37.871	0.193	127	GLY C	-3.176	38.180	3.482
127	GLY D	-2.446	38.830	2.220	128	GLY M	-4.121	37.443	2.222
129	GLY CA	-4.678	37.496	3.642	128	GLY C	-4.644	36.838	4.104
129	GLY D	-4.983	38.158	3.276	129	PRD M	-4.519	39.887	3.482
129	PRD CA	-4.671	34.523	8.998	129	PRD C	-6.316	34.884	4.082
129	PRD D	-6.338	32.887	6.303	129	PRD C8	-4.860	34.684	7.384
129	PRD CG	-4.419	36.116	7.727	129	PRD CD	-4.239	36.870	6.418
130	STR M	-7.031	35.013	5.912	130	STR CA	-8.670	34.611	4.023
130	STR C	-9.218	34.884	4.726	130	STR D	-8.949	35.381	4.029
130	STR C8	-9.049	35.353	7.216	130	STR D0	-9.723	34.624	8.493
131	GLY M	-10.003	33.967	4.349	131	GLY CA	-10.824	34.229	3.074
131	GLY C	-12.205	34.713	3.847	131	GLY D	-12.495	34.722	4.781
132	STR M	-13.040	33.058	2.894	132	STR CA	-14.607	34.433	3.011
132	STR C	-15.219	34.803	1.936	132	STR D	-14.708	34.588	8.824
132	STR C8	-14.590	36.927	3.143	132	STR D0	-14.693	37.539	1.873
133	ALA M	-16.847	34.588	2.294	133	ALA CA	-17.507	34.857	1.324
133	ALA C	-17.630	34.965	8.097	133	ALA D	-17.743	34.437	-1.016
133	ALA C8	-18.866	33.928	1.996	134	ALA M	-17.683	36.288	8.204
134	ALA CA	-17.872	37.239	-0.702	134	ALA C	-18.638	37.369	-1.674
134	ALA D	-16.781	37.585	-2.849	134	ALA C8	-18.263	38.600	-0.187
135	LEU M	-15.478	37.229	-2.046	135	LEU CA	-14.107	37.244	-1.804
135	LEU C	-14.138	36.003	-2.703	135	LEU D	-13.784	36.826	-3.890
135	LEU C8	-13.038	37.328	-0.798	135	LEU CG	-11.693	37.130	-1.388
135	LEU CD1	-11.460	38.413	-2.292	135	LEU CD2	-10.882	36.807	-0.519
136	LVS M	-14.109	36.823	-2.173	136	LVS CA	-14.543	33.597	-3.013
136	LVS C	-18.344	33.739	-4.150	136	LVS C	-19.279	33.431	-5.383
136	LVS C8	-14.903	32.341	-2.186	136	LVS CG	-14.743	31.067	-3.043
136	LVS CD	-15.083	29.892	-2.134	136	LVS CE	-15.743	28.707	-2.778
136	LVS M1	-15.308	28.411	-4.160	137	ALA M	-16.744	34.260	-3.847
137	ALA CA	-17.793	34.416	-4.883	137	ALA C	-17.338	35.303	-6.043
137	ALA D	-17.703	35.049	-7.208	137	ALA C8	-19.094	34.941	-4.243
138	ALA M	-16.929	36.301	-5.729	138	ALA CA	-16.801	37.311	-6.681
138	ALA C	-14.903	36.696	-7.557	138	ALA D	-14.985	36.843	-8.762
138	ALA C8	-15.522	38.967	-3.934	139	VAL M	-13.959	35.959	-7.827
139	VAL CA	-12.946	35.291	-7.837	139	VAL C	-13.423	34.228	-8.720
139	VAL D	-13.208	34.070	-9.877	139	VAL C8	-11.830	34.671	-6.968
139	VAL CG1	-10.919	33.836	-7.866	139	VAL CG2	-11.079	33.780	-6.253
140	ASP M	-14.993	33.336	-8.122	140	ASP CA	-15.274	32.496	-8.929
140	ASP C	-16.823	33.131	-10.084	140	ASP D	-16.980	32.579	-11.190
140	ASP C8	-16.149	31.349	-8.133	140	ASP CG	-15.388	30.640	-7.184
140	ASP CD1	-14.178	30.403	-7.282	141	ASP CD2	-16.139	30.132	-6.329
141	LVS M	-16.638	34.263	-9.820	141	LVS CA	-17.373	35.006	-10.868
141	LVS C	-18.373	35.413	-11.046	141	LVS D	-16.700	35.248	-13.131
141	LVS C8	-18.839	36.275	-10.323	141	LVS CG	-18.884	37.036	-11.306
141	LVS CD	-19.886	38.187	-10.536	141	LVS CE	-20.572	39.031	-11.230
141	LVS M1	-21.138	40.037	-10.273	142	ALA M	-19.167	35.948	-11.966
142	ALA CA	-16.173	36.192	-12.614	142	ALA C	-13.818	35.010	-13.521
142	ALA D	-13.770	35.169	-14.735	142	ALA C8	-12.878	34.697	-11.948
143	VAL M	-13.582	33.886	-12.832	143	VAL CA	-13.168	32.703	-13.630
143	VAL C	-14.346	32.233	-14.696	143	VAL D	-14.140	31.886	-15.639
143	VAL C8	-12.931	31.673	-12.714	143	VAL CD1	-12.300	38.370	-13.461
143	VAL CG1	-11.303	32.195	-12.014	144	ALA M	-13.531	32.338	-13.573
144	ALA CA	-16.764	31.836	-14.041	144	ALA C	-16.920	32.881	-15.861

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144	ALA C	-17.38C	32.263	-16.933	144	ALA C	-17.942	31.968	-13.700
145	SEA M	-16.307	33.948	-15.704	145	SEA C	-16.482	34.917	-14.704
146	SEA C	-15.689	34.773	-17.829	146	SEA D	-15.918	35.321	-18.893
147	SEA C	-17.016	34.776	-16.414	147	SEA C	-15.882	36.935	-15.849
148	GLV M	-14.577	33.934	-17.563	148	GLV C	-13.619	34.286	-19.266
149	GLV C	-12.273	34.491	-18.385	149	GLV D	-11.420	35.856	-16.912
150	VAL M	-12.150	35.142	-17.254	150	VAL C	-10.874	37.091	-15.486
151	VAL C	-9.850	34.836	-16.223	151	VAL D	-10.171	38.001	-15.870
152	VAL C	-11.152	36.977	-15.890	152	VAL C	-9.896	39.001	-16.683
153	VAL C	-12.340	37.913	-16.230	153	VAL C	-8.583	40.126	-16.958
154	VAL C	-7.482	34.230	-16.008	154	VAL C	-7.157	41.432	-18.262
155	VAL C	-6.942	36.133	-16.750	155	VAL C	-6.273	42.965	-12.249
156	VAL C	-5.079	33.483	-16.281	156	VAL C	-5.990	44.173	-11.459
157	VAL C	-7.298	34.355	-13.931	157	VAL C	-4.987	45.419	-10.000
158	VAL C	-8.700	34.385	-11.613	158	VAL C	-4.624	46.807	-4.296
159	VAL C	-8.224	36.890	-12.319	159	VAL C	-3.993	48.320	-0.968
160	VAL C	-9.456	35.386	-12.096	160	VAL C	-3.893	49.967	0.590
161	VAL C	-3.393	34.987	-10.901	161	VAL C	-3.732	51.748	3.462
162	VAL C	-0.973	36.778	-9.490	162	VAL C	-2.673	53.663	8.022
163	ALA M	-2.568	34.633	-11.661	163	ALA C	-2.361	55.711	13.100
164	ALA C	-1.080	34.946	-8.993	164	ALA C	-0.618	57.889	20.967
165	ALA C	-3.537	35.836	-6.657	165	ALA C	0.490	60.197	28.803
166	ALA C	0.714	35.390	-4.207	166	ALA C	0.384	62.720	36.607
167	ALA C	-8.728	34.666	-2.132	167	ALA C	0.840	65.352	44.296
168	ALA C	1.125	33.302	-3.912	168	ALA C	0.317	68.097	51.999
169	ALA C	0.931	32.725	-1.911	169	ALA C	1.827	70.967	59.999
170	GLY C	3.750	31.030	-3.193	170	GLY M	0.319	73.967	67.999
171	GLY D	2.043	34.231	0.123	171	GLY C	0.923	77.097	76.097
172	ASH C	4.189	33.267	-0.118	172	ASH M	0.399	80.348	84.348
173	ASH D	0.344	34.787	2.037	173	ASH C	0.808	83.720	91.720
174	ASH C	4.101	34.829	4.295	174	ASH C	0.123	87.220	99.220
175	ASH D	5.890	36.702	0.300	175	ASH C	0.123	90.848	106.848
176	ASH D	8.494	37.965	0.392	176	ASH C	0.123	94.597	114.597
177	GLU C	4.633	32.537	4.976	177	GLU M	0.711	98.467	122.467
178	GLU D	5.374	30.637	4.222	178	GLU C	0.922	102.467	130.467
179	GLU C	2.493	32.442	6.361	179	GLU C	0.922	106.597	138.597
180	GLU D	1.744	34.322	8.312	180	GLU C	0.922	110.748	146.748
181	GLY M	0.389	31.037	4.227	181	GLU C	0.922	114.917	154.917
182	GLY C	0.389	28.622	4.553	182	GLY C	0.922	119.107	163.107
183	THR M	6.303	27.793	5.382	183	GLY C	0.922	123.317	171.317
184	THR C	7.147	25.487	6.217	184	THR C	0.922	127.548	179.548
185	THR C	8.707	23.335	7.052	185	THR C	0.922	131.797	187.797
186	THR C	6.952	21.904	7.977	186	THR C	0.922	136.067	195.067
187	THR C	6.479	20.210	8.895	187	THR C	0.922	140.357	203.357
188	SEA D	3.141	19.777	9.804	188	SEA C	0.922	144.667	211.667
189	SEA C	4.833	18.028	10.704	189	SEA C	0.922	148.997	219.997
190	SEA C	3.339	16.829	11.594	190	SEA C	0.922	153.348	228.348
191	GLY C	8.434	15.777	12.474	191	SEA C	0.922	157.717	236.717
192	SEA C	4.808	14.777	13.344	192	SEA C	0.922	162.107	245.107
193	SEA C	2.484	13.829	14.204	193	SEA C	0.922	166.517	253.517
194	SEA C	0.496	12.929	15.054	194	SEA C	0.922	170.937	261.937
195	SEA C	0.496	12.029	15.894	195	SEA C	0.922	175.367	270.367
196	SEA C	1.834	11.129	16.714	196	SEA C	0.922	179.807	278.807
197	SEA C	0.167	10.229	17.514	197	SEA C	0.922	184.257	287.257
198	SEA C	1.533	9.329	18.294	198	SEA C	0.922	188.717	295.717
199	SEA C	0.104	8.429	19.054	199	SEA C	0.922	193.187	304.187
200	SEA C	-0.611	7.529	19.794	200	SEA C	0.922	197.667	312.667
201	SEA C	-1.878	6.629	20.514	201	SEA C	0.922	202.157	321.157
202	SEA C	-1.992	5.729	21.214	202	SEA C	0.922	206.657	329.657
203	SEA C	0.499	4.829	21.894	203	SEA C	0.922	211.167	338.167
204	SEA C	0.499	3.929	22.554	204	SEA C	0.922	215.687	346.687
205	SEA C	0.499	3.029	23.194	205	SEA C	0.922	220.217	355.217
206	SEA C	0.499	2.129	23.814	206	SEA C	0.922	224.757	363.757
207	SEA C	0.499	1.229	24.414	207	SEA C	0.922	229.307	372.307
208	SEA C	0.499	0.329	25.004	208	SEA C	0.922	233.867	380.867
209	SEA C	0.499	-0.579	25.574	209	SEA C	0.922	238.437	389.437
210	SEA C	0.499	-1.479	26.124	210	SEA C	0.922	243.017	398.017
211	SEA C	0.499	-2.379	26.654	211	SEA C	0.922	247.607	406.607
212	SEA C	0.499	-3.279	27.164	212	SEA C	0.922	252.207	415.207
213	SEA C	0.499	-4.179	27.654	213	SEA C	0.922	256.817	423.817
214	SEA C	0.499	-5.079	28.124	214	SEA C	0.922	261.437	432.437
215	SEA C	0.499	-5.979	28.574	215	SEA C	0.922	266.067	441.067
216	SEA C	0.499	-6.879	29.004	216	SEA C	0.922	270.707	449.707
217	SEA C	0.499	-7.779	29.414	217	SEA C	0.922	275.357	458.357
218	SEA C	0.499	-8.679	29.804	218	SEA C	0.922	280.017	467.017
219	SEA C	0.499	-9.579	30.174	219	SEA C	0.922	284.687	475.687
220	SEA C	0.499	-10.479	30.524	220	SEA C	0.922	289.367	484.367
221	SEA C	0.499	-11.379	30.854	221	SEA C	0.922	294.057	493.057
222	SEA C	0.499	-12.279	31.164	222	SEA C	0.922	298.757	501.757
223	SEA C	0.499	-13.179	31.454	223	SEA C	0.922	303.467	510.467
224	SEA C	0.499	-14.079	31.724	224	SEA C	0.922	308.187	519.187
225	SEA C	0.499	-14.979	32.004	225	SEA C	0.922	312.917	527.917
226	SEA C	0.499	-15.879	32.264	226	SEA C	0.922	317.657	536.657
227	SEA C	0.499	-16.779	32.504	227	SEA C	0.922	322.407	545.407
228	SEA C	0.499	-17.679	32.724	228	SEA C	0.922	327.167	554.167
229	SEA C	0.499	-18.579	32.924	229	SEA C	0.922	331.937	562.937
230	SEA C	0.499	-19.479	33.104	230	SEA C	0.922	336.717	571.717
231	SEA C	0.499	-20.379	33.264	231	SEA C	0.922	341.507	580.507
232	SEA C	0.499	-21.279	33.404	232	SEA C	0.922	346.307	589.307
233	SEA C	0.499	-22.179	33.524	233	SEA C	0.922	351.117	598.117
234	SEA C	0.499	-23.079	33.624	234	SEA C	0.922	355.937	606.937
235	SEA C	0.499	-23.979	33.704	235	SEA C	0.922	360.767	615.767
236	SEA C	0.499	-24.879	33.764	236	SEA C	0.922	365.607	624.607
237	SEA C	0.499	-25.779	33.804	237	SEA C	0.922	370.457	633.457
238	SEA C	0.499	-26.679	33.824	238	SEA C	0.922	375.317	642.317
239	SEA C	0.499	-27.579	33.824	239	SEA C	0.922	380.187	651.187
240	SEA C	0.499	-28.479	33.804	240	SEA C	0.922	385.067	660.067
241	SEA C	0.499	-29.379	33.764	241	SEA C	0.922	389.957	668.957
242	SEA C	0.499	-30.279	33.704	242	SEA C	0.922	394.857	677.857
243	SEA C	0.499	-31.179	33.624	243	SEA C	0.922	399.767	686.767
244	SEA C	0.499	-32.079	33.524	244	SEA C	0.922	404.687	695.687
245	SEA C	0.499	-32.979	33.404	245	SEA C	0.922	409.617	704.617
246	SEA C	0.499	-33.879	33.264	246	SEA C	0.922	414.557	713.557
247	SEA C	0.499	-34.779	33.104	247	SEA C	0.922	419.507	722.507
248	SEA C	0.499	-35.679	32.924	248	SEA C	0.922	424.467	731.467
249	SEA C	0.499	-36.579	32.724	249	SEA C	0.922	429.437	740.437
250	SEA C	0.499	-37.479	32.504	250	SEA C	0.922	434.417	749.417
251	SEA C	0.499	-38.379	32.264	251	SEA C	0.922	439.407	758.407
252	SEA C	0.499	-39.279	32.004	252	SEA C	0.922	444.407	767.407
253	SEA C	0.499	-40.179	31.724	253	SEA C	0.922	449.417	776.417
254	SEA C	0.499	-41.079	31.424	254	SEA C	0.922	454.437	785.437
255	SEA C	0.499	-41.979	31.104	255	SEA C	0.922	459.467	794.467
256	SEA C	0.499	-42.879	30.764	256	SEA C	0.922	464.507	803.507
257	SEA C	0.499	-43.779	30.404	257	SEA C	0.922	469.557	812.557
258	SEA C	0.499	-44.679	30.024	258	SEA C	0.922	474.617	821.617
259	SEA C	0.499	-45.579	29.624	259	SEA C	0.922	479.687	830.687
260	SEA C	0.499	-46.479	29.204	260	SEA C	0.922	484.767	839.767
261	SEA C	0.499	-47.379	28.764	261	SEA C	0.922	489.857	848.857
262	SEA C	0.499	-48.279	28.304	262	SEA C	0.922	494.957	857.957
263	SEA C	0.499	-49.179	27.824	263	SEA C	0.922	500.067	867.067
264	SEA C	0.499	-50.079	27.324	264	SEA C	0.922	505.187	876.187
265	SEA C	0.499	-50.979	26.804	265	SEA C	0.922	510.317	885.317
266	SEA C	0.499							

169	VAL C0	-1.339	28.624	-8.361	169	VAL C01	-1.947	29.367	-1.374
169	VAL C02	-0.210	27.716	-8.695	169	GLY M	-1.910	21.021	1.126
169	GLY CA	-2.943	32.778	1.626	169	GLY C	-6.098	32.030	0.617
169	GLY D	-6.124	32.104	-8.396	169	TYR M	-5.034	33.730	0.970
169	TYR CA	-6.223	34.046	8.113	169	TYR C	-1.903	29.300	-8.686
169	TYR D	-8.474	36.203	8.084	169	TYR C0	-7.464	34.232	0.964
169	TYR C0	-7.791	32.064	1.709	169	TYR C01	-7.288	32.703	2.947
169	TYR C02	-8.710	32.116	1.133	169	TYR C01	-7.547	31.520	3.610
169	TYR C02	-9.068	30.953	1.809	169	TYR C01	-8.484	30.671	3.848
169	TYR D	-8.886	29.481	1.658	169	PRO M	-6.380	31.499	-1.030
169	PRO C0	-6.943	36.376	-3.938	169	PRO C0	-6.273	36.752	-2.624
169	PRO C0	-7.964	31.344	-3.508	169	PRO CA	-7.134	36.437	-2.560
169	PRO C0	-8.398	33.336	-3.270	169	PRO D	-7.097	32.520	-3.912
169	GLY M	-5.086	33.193	-3.189	169	GLY CA	-4.446	32.977	-3.927
169	GLY C	-4.937	30.702	-3.470	169	GLY D	-4.880	29.733	-4.249
169	LYS M	-3.402	30.579	-2.293	169	LYS CA	-3.856	29.263	-1.743
169	LYS C	-7.033	28.773	-2.516	169	LYS D	-7.308	27.554	-2.624
169	LYS C0	-6.246	29.294	-8.284	169	LYS C0	-5.795	28.106	0.883
169	LYS C0	-6.230	22.289	2.031	169	LYS C0	-5.721	27.271	3.029
169	LYS M1	-4.259	27.463	3.213	169	TYR M	-7.838	29.616	-3.168
169	TYR CA	-9.012	29.043	-3.859	169	TYR C	-8.483	28.300	-3.113
169	TYR D	-7.760	28.714	-5.928	169	TYR C0	-9.942	30.224	-4.242
169	TYR C0	-10.497	30.984	-3.047	169	TYR C01	-11.060	30.303	-1.982
169	TYR C02	-10.496	32.374	-3.026	169	TYR C01	-11.320	31.003	-0.867
169	TYR C02	-10.943	33.088	-1.934	169	TYR C01	-11.520	32.398	-0.886
169	TYR D	-12.008	33.119	0.170	169	TYR C01	-9.297	27.204	-5.374
169	PRO CA	-9.093	26.417	-6.396	169	PRO M	-9.333	27.194	-7.909
169	PRO D	-8.325	26.784	-8.881	169	PRO C0	-10.167	28.329	-6.513
169	PRO C0	-10.450	26.271	-8.094	169	PRO C0	-10.364	26.469	-4.516
169	SER M	-10.097	28.167	-8.019	169	PRO CD	-10.720	28.818	-9.330
169	SER C	-9.025	29.773	-9.593	169	SER CA	-8.946	30.233	-10.762
169	SER CA	-11.524	29.623	-9.481	169	SER D	-7.033	30.846	-8.406
169	VAL M	-8.162	29.944	-8.414	169	SER DG	-5.612	30.891	-8.855
169	VAL C	-5.754	30.131	-9.068	169	VAL CA	-5.796	29.152	-8.944
169	VAL C0	-6.899	31.775	-7.396	169	VAL D	-4.911	32.037	-7.617
169	VAL C02	-8.220	32.303	-7.323	169	VAL C01	-2.714	30.729	-9.883
169	ILE CA	-3.549	30.196	-10.024	169	ILE M	-2.993	30.734	-8.894
169	ILE D	-2.450	31.938	-8.933	169	ILE C	-1.481	30.524	-11.619
169	ILE C0	-3.857	29.978	-12.524	169	ILE C0	-2.220	30.008	-11.512
169	ILE C01	-3.692	30.529	-13.946	169	ILE C02	8.120	30.301	-7.925
169	ALA CA	-1.335	30.517	-6.870	169	ALA M	-1.639	29.838	-8.541
169	ALA D	0.433	29.238	-7.838	169	ALA C	2.261	31.834	-7.636
169	VAL M	0.864	31.430	-7.180	169	VAL CA	3.178	32.457	-5.721
169	VAL C	3.225	31.693	-6.473	169	VAL D	3.842	32.667	-9.392
169	VAL C0	2.439	32.607	-8.753	169	VAL C01	4.077	30.694	-6.398
169	VAL C02	1.374	32.552	-9.043	169	GLY M	6.446	31.233	-6.874
169	GLY CA	5.168	30.703	-5.339	169	GLY C	7.812	31.467	-5.287
169	GLY D	6.491	31.433	-7.286	169	ALA M	9.929	31.099	-5.779
169	ALA CA	8.715	32.037	-5.259	169	ALA C	9.825	30.251	-4.973
169	ALA C	10.198	30.481	-4.719	169	VAL C0	11.970	30.482	-4.981
169	VAL M	10.639	31.162	-6.085	169	VAL CA	12.712	32.691	-7.627
169	VAL C	13.048	31.583	-7.171	169	VAL D	11.271	28.251	-7.833
169	VAL C0	12.075	29.514	-8.166	169	VAL C01	14.267	31.203	-4.900
169	VAL C02	11.675	30.120	-9.300	169	ASP M	15.942	31.804	-5.462
169	ASP CA	10.431	32.100	-7.099	169	ASP C	16.446	31.921	-5.914
169	ASP D	15.339	31.890	-9.292	169	ASP C0	17.103	29.789	-6.972
169	ASP C0	17.120	30.534	-4.887	169	ASP C01	17.087	32.386	-8.047
169	ASP C02	17.680	30.296	-10.191	169	SER M	18.193	30.817	-10.466
169	SER CA	17.622	32.214	-11.670	169	SER C	18.678	33.713	-16.464
169	SER D	18.363	30.492	-9.464	169	SER M	18.258	29.042	-9.423
169	SER DG	18.016	34.961	-9.397	169	SER C	17.581	27.614	-9.947
169	SER CA	18.714	28.665	-9.397	169	SER C0	19.256	28.323	-8.807
169	SER D	17.039	26.413	-9.397					

183	SEN SC	28.589	28.615	-0.231	194	ASN M	16.373	28.094	-0.002
184	ASN CA	25.144	27.317	-0.550	194	ASN C	14.931	26.720	-0.197
184	ASN O	14.138	25.789	-0.097	194	ASN C9	15.014	26.341	-10.722
184	ASN CE	16.990	26.098	-12.076	194	ASN C21	14.780	28.104	-12.277
185	GLN MD2	15.352	26.210	-13.076	195	GLN M	15.542	27.247	-7.159
185	GLN CA	15.276	24.846	-5.035	195	GLN C	14.280	27.494	-5.293
185	GLN O	14.189	25.724	-5.394	195	GLN C9	16.599	26.569	-5.191
185	GLN CE	16.539	26.242	-3.614	195	GLN C2	18.011	26.162	-3.294
185	GLN CE2	18.864	25.799	-4.061	195	GLN CE2	18.266	26.386	-1.934
186	ARC M	12.278	26.959	-4.443	196	ARC CA	12.185	27.774	-3.041
186	ARC C	12.780	26.782	-2.866	196	ARC O	13.698	28.384	-1.093
186	ARC C9	11.313	26.843	-3.116	196	ARC C9	10.214	27.471	-2.161
186	ARC C2	9.467	26.337	-1.668	196	ARC CE	9.866	26.333	-0.117
186	ARC CE	9.961	26.870	1.039	196	ARC CE2	9.367	27.880	1.488
186	ARC CE2	10.066	26.321	1.783	197	ALA M	12.294	30.009	-2.053
187	ALA CA	12.728	31.064	-1.095	197	ALA C	12.262	30.604	-0.517
187	ALA O	11.151	30.043	-0.397	197	ALA C2	12.144	32.402	-2.344
188	SEN M	13.531	30.770	0.549	198	SEN CA	12.671	30.266	1.068
188	SEN C	11.336	30.847	2.412	198	SEN O	10.740	30.111	3.212
188	SEN C9	13.767	30.456	2.937	198	SEN C2	14.137	31.826	2.041
189	PME M	10.043	32.010	1.974	198	PME CA	9.697	32.688	2.418
189	PME C	8.499	32.198	1.609	199	PME O	7.389	32.556	2.011
189	PME C9	9.787	34.217	2.343	199	PME C9	10.317	34.694	0.967
189	PME C2	9.147	34.830	-0.121	199	PME C2	11.619	35.116	0.567
189	PME C2	9.683	35.187	-1.411	199	PME CE2	11.749	35.543	-0.781
190	SEN CA	10.786	35.586	-1.725	199	SEN M	8.703	31.524	0.499
190	SEN O	7.424	31.094	-0.391	199	SEN C	6.663	30.162	0.328
190	SEN C9	7.934	29.023	0.666	199	SEN C9	8.181	30.590	-1.788
190	SEN C2	7.136	30.337	-2.618	199	SEN C2	8.388	30.931	0.326
191	SEN CA	4.341	29.674	0.987	199	SEN C9	4.261	28.330	0.223
191	SEN O	4.543	28.268	-0.095	199	SEN C2	3.015	30.411	0.911
191	SEN C9	2.729	31.285	1.954	199	SEN C2	3.754	27.310	0.928
192	VAL CA	3.623	25.032	0.391	199	VAL M	2.254	25.291	0.666
192	VAL O	1.599	25.698	1.398	199	VAL C	4.701	25.127	1.088
192	VAL C9	4.144	25.727	0.722	199	VAL C9	4.617	25.104	2.592
192	VAL C2	1.938	24.172	0.047	199	VAL C2	0.629	25.564	0.610
193	GLY M	0.081	23.029	-0.901	199	GLY O	0.530	23.244	-2.015
193	GLY C	-1.023	22.289	-0.722	199	PRO CA	-1.662	21.681	-1.073
193	PRO C	-2.237	22.605	-2.914	199	PRO O	-2.403	22.244	-0.085
193	PRO C9	-2.749	20.783	-1.210	199	PRO C9	-2.311	20.622	0.213
193	PRO C2	-1.633	21.954	0.578	199	GLU M	-2.022	23.793	-2.439
193	GLU CA	-3.145	24.090	-3.282	199	GLU C	-2.093	23.631	-0.055
193	GLU O	-2.816	24.398	-4.936	199	GLU C9	-4.043	25.786	-2.670
193	GLU CE	-4.942	25.134	-1.435	199	GLU C2	-4.315	24.860	-0.100
193	GLU CE2	-3.110	24.960	0.163	199	GLU CE2	-5.130	24.520	0.783
194	LEU M	-0.829	25.264	-3.070	199	LEU CA	0.241	25.929	-4.664
194	LEU C	0.228	25.376	-4.059	199	LEU O	0.305	24.121	-4.183
194	LEU C9	1.340	25.739	-3.064	199	LEU C9	2.770	26.178	-4.643
194	LEU C2	2.739	27.716	-4.639	199	LEU C2	0.027	25.721	-3.911
197	ASP M	0.140	24.208	-7.093	197	ASP CA	0.032	25.774	-5.480
197	ASP C	1.307	25.738	-9.293	197	ASP O	1.033	24.734	-9.914
197	ASP C9	-1.067	26.598	-9.191	197	ASP C9	-2.404	26.391	-5.569
197	ASP C2	-2.004	25.155	-8.354	197	ASP C2	-3.035	27.327	-5.088
198	VAL M	2.013	26.889	-9.344	198	VAL CA	3.206	26.970	-10.209
198	VAL C	4.157	27.950	-9.314	198	VAL O	3.752	28.099	-9.587
198	VAL C9	2.894	27.476	-11.637	198	VAL C9	1.930	26.716	-12.937
198	VAL C2	1.337	28.019	-11.684	198	VAL C2	0.274	27.916	-10.016
199	MEY CA	6.439	28.802	-9.698	199	MEY M	6.049	29.810	-10.578
199	MEY O	6.696	29.318	-11.793	199	MEY C	7.660	27.770	-9.077
199	MEY C9	7.363	24.840	-8.139	199	MEY C9	6.753	27.449	-6.568
199	MEY C2	8.227	27.733	-5.587	200	ALA M	7.426	30.942	-10.103
200	ALA CA	7.991	31.024	-11.058	200	ALA C	9.088	32.646	-10.272
200	ALA O	9.127	32.024	-9.060	200	ALA C9	6.032	32.070	-11.638

201	PRC H	9.927	23.499	-13.953	201	PRC CA	11.013	34.130	-13.230
201	PRD C	10.450	25.127	-9.230	201	PRC D	9.579	31.007	-9.492
201	PRD CD	11.017	26.723	-11.400	201	PRC CC	11.392	34.040	-17.670
201	PRD CD	9.941	23.410	-12.403	201	GLV H	10.925	29.204	-1.021
202	GLV CA	10.473	26.234	-7.044	202	GLV C	11.390	26.495	-6.315
202	GLV D	11.312	27.124	-4.979	202	VAL H	12.015	26.303	-6.613
202	VAL CA	13.049	26.929	-3.710	202	VAL C	14.706	26.017	-6.469
202	VAL C	13.133	27.731	-7.503	202	VAL CC	14.014	23.000	-5.351
202	VAL CC1	14.098	26.106	-6.612	202	VAL CC2	14.079	24.741	-4.178
204	SRH H	14.865	29.102	-3.859	204	SRH CA	15.572	40.201	-4.487
204	SRH C	15.047	40.610	-7.872	204	SRH D	15.706	40.605	-3.889
204	SRH CD	17.017	29.974	-6.324	204	SRH CC	17.732	41.126	-4.672
205	SLF H	13.771	42.065	-8.008	205	SLF CA	13.869	41.234	-9.225
205	SLF C	13.207	42.749	-9.478	205	SLF D	12.675	39.498	-3.648
205	SLF CC	11.832	40.833	-9.144	205	SLF CC1	11.436	39.396	-3.810
205	SLF CC2	10.899	41.281	-10.467	205	SLF CC1	12.257	38.412	-9.771
206	GLN H	13.956	43.093	-10.409	206	GLN CA	14.204	44.317	-10.834
206	GLN C	13.002	44.978	-11.630	206	GLN D	12.669	44.318	-12.621
206	GLN CD	17.205	44.708	-11.740	206	GLN CC	14.684	44.103	-10.980
206	GLN CC2	14.536	45.145	-10.607	206	GLN CC1	18.328	44.936	-9.353
207	SRH CA	11.217	46.260	-9.857	207	SRH H	12.359	46.864	-11.214
207	SRH D	11.910	46.571	-11.987	207	SRH C	11.099	46.093	-11.749
207	SRH CC	8.993	46.637	-11.004	207	SRH CC	9.910	45.053	-11.569
208	TMR CC2	9.171	46.036	-12.613	208	TMR H	10.054	48.664	-12.326
208	TMR C	8.620	50.339	-14.754	208	TMR DC1	7.570	49.414	-13.146
208	TMR D	9.107	50.415	-13.357	208	TMR CA	9.675	50.092	-12.173
208	LEU H	9.636	50.488	-10.803	208	TMR D	8.423	49.807	-10.049
208	LEU C	8.673	51.613	-10.228	208	LEU CA	9.192	52.158	-8.959
208	LEU CD	10.335	52.610	-9.262	208	LEU D	9.140	54.227	-10.222
208	LEU CD1	11.968	52.192	-7.938	208	LEU CC	10.004	50.816	-7.616
210	PRD H	7.700	51.114	-6.472	208	LEU CD2	9.607	50.282	-6.649
210	PRD C	8.383	54.139	-8.444	210	PRD CA	7.273	55.517	-8.649
210	PRD CD	8.302	56.573	-8.639	210	PRC D	9.491	56.645	-8.104
210	PRD CD	7.193	55.733	-7.517	210	PRC CC	6.004	54.379	-6.944
211	GLV CA	9.049	53.491	-7.271	211	SLV H	8.077	57.065	-9.353
211	GLV D	11.176	58.763	-9.410	211	SLV C	10.094	58.454	-10.495
212	ASH CA	10.903	59.005	-10.259	212	ASH H	9.851	57.770	-11.587
212	ASH C	11.188	57.422	-12.043	212	ASH C	12.039	56.753	-12.056
212	ASH CC	11.803	57.181	-12.420	212	ASH CD	11.224	58.393	-13.499
212	ASH ND2	12.273	58.185	-14.814	212	ASH CD1	11.853	57.054	-13.323
213	LVS CA	12.810	59.159	-15.376	213	LVS H	11.803	58.749	-11.247
213	LVS D	11.775	54.946	-10.537	213	LVS C	12.668	53.439	-10.866
213	LVS CC	13.206	53.039	-11.613	213	LVS CD	12.769	53.241	-9.859
213	LVS CC	14.105	56.694	-8.767	213	LVS CD	13.246	57.030	-7.312
214	TVR H	13.681	58.218	-6.870	213	LVS NI	10.049	58.763	-7.921
214	TVR C	14.383	52.703	-10.444	214	TVR CA	13.803	51.246	-10.722
214	TVR CD	14.641	50.650	-9.609	214	TVR D	10.211	51.293	-8.817
214	TVR CD1	14.689	50.981	-11.984	214	TVR CC	14.130	51.621	-13.746
214	TVR CC1	14.230	52.047	-13.678	214	TVR CC2	13.129	51.069	-14.016
214	TVR C2	13.204	53.475	-14.814	214	TVR CC2	12.054	51.609	-15.178
215	GLV H	14.038	52.095	-13.850	214	TVR DM	12.756	53.458	-10.496
215	GLV C	14.130	49.947	-9.158	215	GLV CA	14.622	48.772	-7.905
216	ALA H	14.810	47.323	-7.749	215	GLV D	13.249	46.917	-8.521
216	ALA C	13.602	46.638	-6.831	216	ALA CA	14.454	45.205	-6.781
216	ALA CD	15.715	44.922	-5.912	216	ALA D	13.940	45.527	-4.479
217	TVR CA	11.964	44.354	-6.887	217	TVR H	12.758	42.982	-5.975
217	TVR D	12.252	43.688	-4.440	217	TVR C	12.033	41.028	-4.947
217	TVR CC	10.117	41.642	-9.656	217	TVR CC	10.473	43.862	-4.570
217	TVR CC2	9.016	43.291	-4.214	217	TVR CD1	10.046	45.991	-3.236
217	TVR CC2	8.634	43.933	-4.785	217	TVR CC1	10.439	47.267	-2.790
217	TVR DM	8.953	47.219	-4.381	217	TVR C2	9.358	47.802	-3.391
218	ASH CA	11.646	49.160	-2.938	218	ASH H	11.790	61.396	-3.391
			29.042	-3.227	218	ASH C	10.204	39.436	-2.749

218	ALA D	0.763	42.367	-1.017	218	ALA CB	12.953	30.340	-2.134
218	ALA CC	14.031	39.866	-2.343	218	ALA CD1	14.612	30.709	-3.422
218	ALA WD2	14.660	39.644	-3.365	219	GLV M	0.678	39.934	-3.289
219	GLV CA	0.382	38.132	-2.649	219	GLV C	7.870	37.304	-3.681
219	GLV D	7.873	37.802	-4.876	220	YMR M	4.541	36.438	-3.209
220	YMR CA	3.697	35.936	-4.179	220	YMR C	4.879	37.044	-4.864
220	YMR D	4.417	36.742	-5.918	220	YMR CB	4.823	36.819	-3.526
220	YMR CD1	4.136	35.543	-2.451	220	YMR CD2	9.704	33.696	-2.988
221	SRH M	4.738	38.238	-4.353	221	SRH CA	3.984	39.201	-3.169
221	SRH C	4.760	39.641	-6.383	221	SRH D	4.117	40.208	-7.277
221	SRH CB	3.323	40.383	-4.346	221	SRH DG	3.435	40.282	-3.149
222	MEY M	0.060	39.389	-6.485	222	MEY CE	6.471	42.771	-3.173
222	MEY SD	7.749	41.333	-4.993	222	MEY CC	0.804	41.399	-6.602
222	MEY CB	0.311	40.013	-7.218	222	MEY CA	4.916	39.670	-7.638
222	MEY C	4.877	38.435	-8.947	222	MEY D	7.084	38.567	-9.775
223	ALA M	0.854	37.244	-8.841	223	ALA CA	6.469	36.020	-8.885
223	ALA C	5.200	36.068	-9.707	223	ALA D	3.133	35.948	-10.929
223	ALA CB	6.509	34.807	-7.923	224	SRH M	4.076	34.360	-9.831
224	SRH CA	2.738	36.688	-9.705	224	SRH C	2.463	37.161	-11.039
224	SRH D	2.145	36.893	-12.057	224	SRH CB	1.001	34.995	-8.603
224	SRH DG	0.492	36.895	-9.197	225	PRD M	3.156	31.411	-11.159
225	PRD CA	3.095	39.130	-12.439	225	PRD C	3.764	38.469	-13.624
225	PRD D	3.406	38.650	-14.804	225	PRD CB	3.633	40.311	-12.854
225	PRD CC	4.411	40.402	-10.764	225	PRD CD	3.735	39.324	-10.054
226	WIS M	4.769	37.626	-13.299	226	WIS CA	5.446	34.879	-14.362
226	WIS C	4.418	35.947	-15.061	226	WIS C	4.425	35.809	-16.293
226	WIS CB	6.608	36.046	-13.765	226	WIS CG	7.814	36.359	-13.358
226	WIS WD1	8.948	37.688	-12.170	226	WIS CD2	8.883	37.118	-16.167
226	WIS CD1	9.270	38.932	-12.236	226	WIS ME2	9.771	37.066	-13.643
227	VAL M	3.593	35.366	-14.199	227	VAL CA	2.883	34.388	-14.727
227	VAL C	1.479	35.197	-15.621	227	VAL D	1.018	34.773	-16.490
227	VAL CB	2.103	33.444	-13.619	227	VAL CD1	1.076	32.676	-14.246
227	VAL CD2	3.204	32.665	-12.891	228	ALA M	1.003	36.242	-14.814
228	ALA CA	0.011	37.109	-15.517	228	ALA C	0.343	37.538	-16.068
228	ALA D	-0.253	37.633	-17.828	228	ALA CB	-0.307	38.333	-14.668
229	GLV M	1.793	38.028	-16.941	229	GLV CA	2.352	38.608	-18.239
229	GLV C	2.420	37.197	-19.187	229	GLV D	2.189	37.375	-20.304
230	ALA M	2.713	35.988	-18.646	230	ALA CA	2.794	34.801	-19.546
230	ALA C	1.424	34.800	-20.153	230	ALA D	1.380	34.203	-21.343
230	ALA CB	3.298	33.624	-18.709	231	ALA M	0.385	34.623	-19.328
231	ALA CA	-1.010	34.416	-19.744	231	ALA C	-1.286	35.423	-20.064
231	ALA D	-1.909	35.056	-21.852	231	ALA CB	-1.932	34.664	-18.549
232	ALA M	-0.778	34.637	-20.721	232	ALA CA	-1.013	37.663	-21.792
232	ALA C	-0.281	37.284	-23.078	232	ALA D	-0.841	37.901	-24.187
232	ALA CB	-0.742	39.121	-21.377	233	LEU M	0.938	36.724	-22.967
233	LEU CA	1.617	34.293	-24.209	233	LEU C	0.821	35.169	-24.880
233	LEU D	0.696	33.231	-26.111	233	LEU CB	3.063	35.877	-23.907
233	LEU CC	3.996	36.994	-23.653	233	LEU CD1	5.239	36.342	-22.921
233	LEU CD2	4.741	37.833	-24.680	234	ILE M	0.357	34.199	-24.047
234	ILE CD1	0.306	30.466	-21.657	234	ILE CG1	0.484	31.223	-23.189
234	ILE CB	-0.811	32.014	-23.570	234	ILE CG2	-1.803	30.900	-24.891
234	ILE CA	-0.606	33.076	-24.644	234	ILE C	-1.621	33.597	-25.634
234	ILE D	-1.893	33.144	-26.544	235	LEU M	-2.390	34.463	-24.779
235	LEU CA	-3.396	35.028	-25.623	235	LEU C	-3.258	33.843	-26.072
235	LEU D	-4.109	35.916	-27.589	235	LEU CB	-4.432	35.765	-24.378
235	LEU CC	-5.140	34.899	-23.342	235	LEU CD1	-5.652	35.683	-22.145
235	LEU CD2	-6.252	34.138	-24.120	236	SRH M	-2.094	34.438	-26.798
236	SRH CA	-3.764	37.237	-27.984	236	SRH C	-1.491	36.292	-29.144
236	SRH D	-3.746	36.634	-30.290	236	SRH CB	-0.633	38.234	-27.733
236	SRH DG	0.999	37.571	-27.582	237	LVS M	-1.046	35.067	-28.882
237	LVS CA	-0.846	34.085	-29.952	237	LVS C	-2.113	33.277	-30.249
237	LVS D	-2.378	32.951	-31.444	237	LVS CB	0.272	33.112	-29.591
237	LVS CC	0.677	32.240	-30.716	237	LVS CD	2.920	31.938	-30.662

237	LV5 CE	2.343	30.762	-21.729	237	LV5 M2	3.923	29.048	-21.996
238	MIS M	-2.931	31.909	-20.312	238	MIS CA	-4.168	31.163	-20.370
239	MIS C	-5.334	31.909	-20.697	239	MIS D	-5.713	31.904	-27.862
239	MIS CD	-3.948	30.862	-28.311	239	MIS CE	-5.889	29.921	-29.237
239	MIS CD1	-1.707	29.679	-28.838	239	MIS CD2	-2.137	29.251	-30.394
239	MIS CD3	-1.886	28.891	-29.642	239	MIS M22	-1.949	28.680	-30.399
239	PRD M	-5.848	33.917	-29.368	239	PRD CA	-6.998	34.779	-29.773
239	PRD C	-2.204	34.632	-28.332	239	PRD D	-8.949	34.819	-27.862
239	PRD CD	-7.818	35.977	-29.713	239	PRD CE	-6.666	35.294	-21.827
239	PRD CD	-3.436	34.434	-20.668	239	AS4 M	-3.396	32.969	-29.227
240	AS4 CA	-9.329	32.841	-29.216	240	AS4 C	-0.500	31.180	-27.980
240	AS4 D	-10.340	30.610	-27.576	240	AS4 CB	-9.403	31.249	-30.535
240	AS4 CG	-7.971	30.827	-30.889	240	AS4 CD1	-7.898	31.900	-31.147
240	AS4 CD2	-7.675	29.809	-30.976	241	TMP M	-8.354	31.804	-27.304
241	TMP CA	-6.304	30.124	-26.120	241	TMP C	-9.106	30.438	-24.936
241	TMP D	-9.843	31.833	-24.686	241	TMP CB	-6.979	29.830	-28.470
241	TMP CG	-6.094	28.903	-26.937	241	TMP CD1	-6.308	28.433	-27.810
241	TMP CD2	-6.839	28.324	-26.185	241	TMP CD3	-3.362	27.547	-28.231
241	TMP CE2	-6.414	27.476	-27.216	241	TMP CE3	-4.097	28.406	-24.981
241	TMP C13	-3.193	26.786	-27.174	241	TMP C13	-2.912	27.667	-24.043
241	TMP C42	-2.478	26.873	-26.003	242	TMR M	-9.727	29.781	-24.142
242	TMR CA	-10.488	30.119	-22.911	242	TMR C	-9.669	30.176	-21.747
242	TMR D	-8.333	29.674	-21.937	242	TMR CB	-11.579	29.032	-22.478
242	TMR CD1	-10.837	27.786	-22.474	242	TMR CG2	-12.404	28.907	-23.899
243	AS4 M	-9.946	30.459	-20.611	243	AS4 CD2	-11.787	30.604	-18.747
243	AS4 DD1	-11.465	31.918	-16.788	243	AS4 CG	-11.893	31.331	-17.985
243	AS4 CB	-9.708	31.820	-18.332	243	AS4 CA	-9.853	30.731	-19.644
243	AS4 C	-8.637	29.303	-19.010	243	AS4 D	-7.593	29.136	-18.440
244	TMR M	-9.364	28.362	-19.293	244	TMR CA	-9.381	26.934	-19.839
244	TMR C	-8.133	26.393	-19.802	244	TMR D	-7.374	25.757	-19.111
244	TMR CB	-10.665	26.888	-19.494	244	TMR DD1	-11.738	26.675	-18.684
244	TMR CG2	-10.303	24.595	-19.157	245	GLN M	-8.082	26.716	-21.073
245	GLN CA	-6.964	26.362	-21.962	245	GLN C	-5.647	27.820	-21.520
245	GLN D	-4.373	26.393	-21.447	245	GLN CB	-7.330	26.999	-23.397
245	GLN CG	-8.265	25.326	-23.989	245	GLN CD	-5.493	25.873	-25.428
245	GLN DD1	-9.306	26.769	-23.727	245	GLN ME2	-7.745	25.312	-26.370
246	VAL M	-5.697	28.304	-21.218	246	VAL CA	-4.677	29.040	-20.770
246	VAL C	-3.936	26.462	-19.467	246	VAL D	-2.789	28.227	-19.361
246	VAL CB	-4.779	30.555	-20.621	246	VAL CG1	-3.544	31.272	-20.827
246	VAL CG2	-5.168	31.138	-21.959	247	ARC M	-4.767	28.240	-18.462
247	ARC CA	-4.380	27.714	-17.168	247	ARC C	-3.770	26.292	-17.360
247	ARC D	-3.763	25.985	-16.764	247	ARC CB	-3.533	27.667	-16.149
247	ARC CG	-4.987	27.895	-16.852	247	ARC CD	-6.856	27.170	-13.793
247	ARC ME	-5.448	26.757	-12.546	247	ARC C1	-5.893	26.846	-11.313
247	ARC M41	-7.064	27.484	-11.210	247	ARC M42	-5.177	26.428	-10.270
248	SER M	-6.480	25.905	-18.131	248	SER CA	-4.839	24.131	-18.426
248	SER C	-2.637	24.886	-19.072	248	SER D	-1.848	23.253	-18.583
248	SER CB	-3.034	23.408	-19.372	248	SER DG	-6.146	23.890	-18.532
249	SER M	-2.500	24.853	-20.136	249	SER CA	-1.223	24.874	-20.851
249	SER C	-0.071	25.307	-19.940	249	SER D	2.826	24.703	-20.049
249	SER CB	-3.369	25.758	-22.068	249	SER DG	-3.300	25.419	-21.956
250	LEU M	-8.209	26.333	-19.160	250	LEU CD2	1.834	29.814	-18.222
250	LEU CD1	-8.373	22.453	-17.268	250	LEU CC	0.352	28.438	-18.151
250	LEU CB	0.178	25.883	-17.903	250	LEU CA	0.718	26.837	-18.216
250	LEU C	1.092	25.894	-17.283	250	LEU C	2.283	25.421	-17.032
251	GLN M	0.068	25.807	-16.714	251	GLN ME2	-2.750	25.512	-12.137
251	GLN DD1	-2.819	23.424	-12.933	251	GLN CD	-3.949	24.850	-13.834
251	GLN CG	-1.218	24.814	-13.994	251	GLN CB	-0.837	23.621	-14.877
251	GLN CA	0.381	23.941	-15.745	251	GLN C	0.919	22.664	-16.361
251	GLN D	1.743	22.014	-13.616	252	AS4 M	0.633	22.394	-17.990
252	AS4 CA	1.092	21.206	-18.782	252	AS4 C	2.394	21.359	-18.991
252	AS4 D	2.809	20.442	-19.768	252	AS4 CB	0.006	20.780	-19.292
252	AS4 CG	-1.034	19.924	-19.573	252	AS4 DD1	-0.836	19.393	-17.582

252	ASN MD2	-2.234	29.834	-19.161	253	TM2 h	3.818	22.803	-18.923
253	TM2 CA	4.234	22.717	-19.713	253	TM2 L	9.381	23.247	-18.811
253	TM2 D	4.348	23.733	-19.427	253	TM2 CB	4.886	23.672	-18.932
253	TM2 CC1	3.993	20.937	-20.428	253	TM2 CC2	3.147	23.130	-22.832
254	TM2 N	5.218	23.177	-17.851	254	TM2 CA	6.214	23.612	-18.581
254	TM2 C	7.466	22.750	-17.612	254	TM2 D	7.402	23.980	-17.891
254	TM2 CB	5.664	23.958	-19.132	254	TM2 CC1	5.129	22.178	-18.040
254	TM2 CC2	4.530	24.849	-14.802	255	TM2 N	8.499	23.796	-14.876
255	TM2 CA	9.771	22.504	-15.817	255	TM2 L	9.621	23.031	-14.414
255	TM2 D	9.439	22.786	-13.474	255	TM2 CB	11.020	23.483	-15.897
255	TM2 CC1	11.032	23.709	-17.921	255	TM2 CC2	12.286	22.628	-15.406
256	LVS h	9.686	20.702	-14.314	256	LVS CA	9.364	20.063	-13.610
256	LVS L	10.512	20.333	-12.063	256	LVS D	11.662	20.274	-12.892
256	LVS CB	9.074	18.990	-13.249	256	LVS CC	9.818	17.805	-11.021
256	LVS CD	10.286	16.948	-11.777	256	LVS CE	10.212	19.940	-10.623
256	LVS M2	9.243	14.969	-11.554	257	LEU h	10.212	20.474	-18.824
257	LEU CA	11.272	21.036	-9.893	257	LEU L	11.250	20.232	-8.616
257	LEU D	12.096	20.865	-7.732	257	LEU CB	11.187	22.947	-9.922
257	LEU CC	11.357	23.420	-10.968	257	LEU CD1	11.245	25.083	-9.921
257	LEU CC2	12.678	23.468	-11.323	258	GLY h	10.431	19.782	-8.208
258	GLY CA	10.602	18.793	-4.879	258	GLY L	9.168	18.703	-6.373
258	GLY D	8.283	18.956	-7.202	259	ASP h	9.824	18.282	-5.190
259	ASP CA	7.757	17.894	-4.916	259	ASP L	6.659	18.941	-4.709
259	ASP D	6.839	20.039	-4.214	259	ASP CB	7.996	17.840	-3.833
259	ASP CC	4.781	17.128	-2.241	259	ASP CD1	8.611	17.327	-2.354
259	ASP CC2	7.098	16.299	-1.321	260	SIR h	5.940	18.610	-5.312
260	SIR CA	4.481	18.587	-5.929	260	SIR L	4.046	20.362	-4.289
260	SIR D	3.300	21.503	-4.446	260	SIR CB	3.345	18.919	-4.289
260	SIR CC	2.743	17.937	-5.448	261	PHE h	4.241	19.778	-3.112
261	PHE CA	3.831	20.468	-1.885	261	PHE L	4.544	21.846	-1.863
261	PHE D	3.944	22.848	-1.432	261	PHE CB	4.053	19.749	-0.963
261	PHE CC	3.349	20.337	0.719	261	PHE CD1	2.206	20.163	1.125
261	PHE CC2	4.403	21.060	1.538	261	PHE CE1	1.737	20.717	2.318
262	TYR h	3.963	21.802	2.748	261	PHE CE2	2.685	21.463	3.114
262	TYR L	5.778	21.768	-2.305	262	TYR CA	4.698	22.914	-2.251
262	TYR D	6.820	23.689	-3.949	262	TYR D	7.201	24.833	-2.293
262	TYR CB	8.122	22.433	-1.831	262	TYR CC	6.146	21.892	-0.454
262	TYR CD1	8.094	20.424	-0.364	262	TYR CD2	8.149	22.648	0.698
262	TYR CE1	8.062	19.973	0.862	262	TYR CE2	8.114	22.069	1.942
262	TYR CE2	8.069	20.672	2.010	262	TYR CM	7.965	20.029	3.205
263	TYR h	6.626	23.104	-4.493	263	TYR CA	4.812	23.653	-8.022
263	TYR L	5.626	23.680	-6.956	263	TYR D	5.781	24.117	-8.111
263	TYR CB	7.928	22.768	-6.681	263	TYR CC	9.279	23.039	-6.068
263	TYR CD1	10.064	24.046	-6.657	263	TYR CD2	9.800	22.342	-4.993
263	TYR CE1	11.333	24.328	-6.168	263	TYR CE2	11.062	22.640	-4.491
263	TYR CE2	11.838	23.618	-5.106	263	TYR DM	13.063	23.949	-4.897
264	GLY h	4.471	23.161	-6.516	264	GLY CA	3.301	23.064	-7.612
264	GLY L	3.847	22.196	-8.534	264	GLY D	4.647	21.274	-8.563
265	LVS h	3.636	22.477	-9.754	265	LVS CA	3.834	21.798	-10.971
265	LVS CB	9.188	22.232	-11.464	265	LVS D	8.894	21.843	-12.384
265	LVS CD	2.753	22.071	-12.044	265	LVS CC	1.490	21.943	-11.303
265	LVS CE	0.710	20.548	-12.879	265	LVS CE2	-5.692	20.496	-11.391
266	GLY h	-3.678	20.757	-12.489	266	GLY L	3.787	23.226	-10.817
266	GLY D	7.120	23.612	-11.323	266	GLY C	7.193	23.032	-11.818
267	LEU CA	6.177	23.793	-11.048	267	LEU h	8.262	23.336	-12.480
267	LEU D	8.490	26.650	-13.097	267	LEU L	7.804	26.773	-14.437
267	LEU CC	7.953	23.909	-19.298	267	LEU CB	10.010	26.935	-13.216
267	LEU CD1	10.432	28.060	-14.058	267	LEU CD2	10.896	29.331	-13.290
267	LEU CD2	11.924	27.921	-14.327	268	ILE h	7.064	27.863	-14.632
268	ILE CA	4.406	28.033	-15.944	268	ILE L	7.426	28.246	-17.063
268	ILE D	8.539	28.793	-16.912	268	ILE CB	8.969	29.210	-15.899
268	ILE CC1	6.099	30.541	-15.552	268	ILE CC2	4.743	28.923	-14.867
268	ILE CD1	8.399	31.745	-16.262	269	ASN h	7.897	27.843	-11.237

269	ALA CA	1.362	27.973	-19.437	269	ALA C	6.839	28.914	-28.493
269	ALA D	5.965	27.760	-21.943	269	ALA C2	0.457	28.613	-28.493
269	ALA C6	5.363	26.826	-21.232	269	ALA HD2	0.493	27.616	-21.122
270	VAL CA	22.013	21.796	-21.472	270	VAL H	4.988	29.868	-28.724
270	VAL D	5.863	21.418	-21.014	270	VAL C	6.889	28.897	-28.054
270	VAL C6	5.857	21.969	-21.172	270	VAL C8	3.496	21.910	-21.422
271	GLN H	6.049	22.797	-21.976	271	VAL C62	6.429	22.363	-22.232
271	GLN C	5.325	29.701	-21.392	271	GLN CA	7.603	29.310	-24.764
271	GLN C8	6.469	27.936	-21.231	271	GLN D	6.213	27.806	-24.891
271	GLN CD	0.104	21.220	-21.964	271	GLN C6	9.486	28.618	-26.236
271	GLN HD2	20.901	28.913	-21.292	271	GLN HD1	31.269	28.970	-27.718
272	ALA CA	21.702	28.913	-21.910	272	ALA H	6.977	28.909	-24.892
272	ALA D	6.224	21.712	-21.140	272	ALA C	6.701	28.958	-24.264
272	ALA H	3.978	21.303	-21.002	272	ALA C8	6.763	26.762	-22.172
272	ALA C	6.247	26.861	-21.135	272	ALA CA	2.740	26.723	-22.659
272	ALA C8	2.081	27.328	-21.020	272	ALA D	0.899	27.219	-24.255
273	ALA CA	2.736	27.773	-21.985	273	ALA H	1.785	28.464	-24.762
273	ALA C	2.932	30.393	-20.210	273	ALA C6	2.189	29.166	-25.667
273	ALA C8	1.730	21.367	-21.090	273	ALA D	0.980	28.949	-27.021
273	GLN H	2.930	27.344	-21.714	273	GLN CA	2.948	28.309	-28.827
273	GLN C	1.147	27.261	-21.777	273	GLN D	2.260	27.807	-21.016
273	GLN OT	2.193	27.361	-20.590	273	GLN C8	0.656	25.724	-21.720
273	GLN C6	0.591	24.864	-27.667	273	GLN CD	-0.023	25.916	-21.632
273	GLN HD1	-1.376	23.873	-20.729	273	GLN HD2	-1.373	25.611	-24.893

The above structural studies together with the kinetic data presented herein and elsewhere (Philipp, M., et al. (1983) Mol. Cell. Biochem. 51, 5-32; Svendsen, I.B. (1976) Carlsberg Res. Comm. 41, 237-291; Markland, S.F. Id; Stauffe, D.C., et al. (1965) J. Biol. Chem. 244, 5333-5338) indicate that the subsites in the binding cleft of subtilisin are capable of interacting with substrate amino acid residues from P-4 to P-2'.

- 10 The most extensively studied of the above residues are Gly166, Gly169 and Ala152. These amino acids were identified as residues within the S-1 subsite. As seen in Fig. 3, which is a stereoview of the S-1 subsite, Gly166 and Gly169 occupy positions at the
15 bottom of the S-1 subsite, whereas Ala152 occupies a position near the top of S-1, close to the catalytic Ser221.

- 20 All 19 amino acid substitutions of Gly166 and Gly169 have been made. As will be indicated in the examples which follow, the preferred replacement amino acids for Gly166 and/or Gly169 will depend on the specific amino acid occupying the P-1 position of a given substrate.

- 25 The only substitutions of Ala152 presently made and analyzed comprise the replacement of Ala152 with Gly and Ser. The results of these substitutions on P-1 specificity will be presented in the examples.

- 30 In addition to those residues specifically associated with specificity for the P-1 substrate amino acid, Tyr104 has been identified as being involved with P-4 specificity. Substitutions at Phe189 and Tyr217,

35

however, are expected to respectively effect P-2' and P-1' specificity.

5 The catalytic activity of subtilisin has also been modified by single amino acid substitutions at Asn155. The catalytic triad of subtilisin is shown in Fig. 4. As can be seen, Ser221, His64 and Asp32 are positioned to facilitate nucleophilic attack by the serine hydroxylate on the carbonyl of the scissile peptide bond. Crystallographic studies of subtilisin (Robertus, *et al.* (1972) Biochem. 11, 4293-4303; Matthews, *et al.* (1975) J. Biol. Chem. 250, 7120-7126; Poulos, *et al.* (1976) J. Biol. Chem. 250, 1097-1103) show that two hydrogen bonds are formed with the oxyanion of the substrate transition state. One
10 hydrogen bond donor is from the catalytic serine-221 main-chain amide while the other is from one of the NE2 protons of the asparagine-155 side chain. See Fig. 4.

20 Asn155 was substituted with Ala, Asp, His, Glu and Thr. These substitutions were made to investigate the the stabilization of the charged tetrahedral intermediate of the transition state complex by the potential hydrogen bond between the side chain of
25 Asn155 and the oxyanion of the intermediate. These particular substitutions caused large decreases in substrate turnover, k_{cat} (200 to 4,000 fold), marginal decreases in substrate binding K_m (up to 7 fold), and a loss in transition state stabilization energy of 2.2
30 to 4.7 kcal/mol. The retention of K_m and the drop in k_{cat} will make these mutant enzymes useful as binding proteins for specific peptide sequences, the nature of which will be determined by the specificity of the precursor protease.
35

Various other amino acid residues have been identified which affect alkaline stability. In some cases, mutants having altered alkaline stability also have altered thermal stability.

5 In B amyloliquefaciens subtilisin residues Asp36, Ile107, Lys170, Ser204 and Lys213 have been identified
10 as residues which upon substitution with a different amino acid alter the alkaline stability of the mutated enzyme as compared to the precursor enzyme. The
15 substitution of Asp36 with Ala and the substitution of Lys170 with Glu each resulted in a mutant enzyme having a lower alkaline stability as compared to the wild type subtilisin. When Ile107 was substituted with Val, Ser204 substituted with Cys, Arg or Leu or
20 Lys213 substituted with Arg, the mutant subtilisin had a greater alkaline stability as compared to the wild type subtilisin. However, the mutant Ser204P demonstrated a decrease in alkaline stability.

20 In addition, other residues, identified as being associated with the modification of other properties of subtilisin, also affect alkaline stability. These
25 residues include Ser24, Met50, Glu156, Gly166, Gly169 and Tyr217. Specifically the following particular substitutions result in an increased alkaline
30 stability: Ser24C, Met50F, Gly156Q or S, Gly166A, H, K, N or Q, Gly169S or A, and Tyr217F, K, R or L. The mutant Met50V, on the other hand, results in a decrease in the alkaline stability of the mutant subtilisin as compared to wild type subtilisin.

35 Other residues involved in alkaline stability based on the alkaline stability screen include Asp197 and Met222. Particular mutants include Asp197(R or A) and Met 222 (all other amino acids).

Various other residues have been identified as being involved in thermal stability as determined by the thermal stability screen herein. These residues include the above identified residues which effect alkaline stability and Met199 and Tyr21. These latter two residues are also believed to be important for alkaline stability. Mutants at these residues include I199 and F21.

The amino acid sequence of B. amyloliquefaciens subtilisin has also been modified by substituting two or more amino acids of the wild-type sequence. Six categories of multiply substituted mutant subtilisin have been identified. The first two categories comprise thermally and oxidatively stable mutants. The next three other categories comprise mutants which combine the useful properties of any of several single mutations of B. amyloliquefaciens subtilisin. The last category comprises mutants which have modified alkaline and/or thermal stability.

The first category comprises double mutants in which two cysteine residues have been substituted at various amino acid residue positions within the subtilisin molecule. Formation of disulfide bridges between the two substituted cysteine residues results in mutant subtilisins with altered thermal stability and catalytic activity. These mutants include A21/C22/C87 and C24/C87 which will be described in more detail in Example 11.

The second category of multiple subtilisin mutants comprises mutants which are stable in the presence of various oxidizing agents such as hydrogen peroxide or peracids. Examples 1 and 2 describe these mutants

which include F50/I124/Q222, F50/I124, F50/Q222, F50/L124/Q222, I124/Q222 and L124/Q222.

5 The third category of multiple subtilisin mutants
comprises mutants with substitutions at position 222
combined with various substitutions at positions 166
or 169. These mutants, for example, combine the
property of oxidative stability of the A222 mutation
with the altered substrate specificity of the various
10 166 or 169 substitutions. Such multiple mutants
include A166/A222, A166/C222, F166/C222, K166/A222,
K166/C222, V166/A222 and V166/C222. The K166/A222
mutant subtilisin, for example, has a kcat/Km ratio
which is approximately two times greater than that of
15 the single A222 mutant subtilisin when compared using
a substrate with phenylalanine as the P-1 amino acid.
This category of multiple mutant is described in more
detail in Example 12.

20 The fourth category of multiple mutants combines
substitutions at position 156 (Glu to Q or S) with the
substitution of Lys at position 166. Either of these
single mutations improve enzyme performance upon
substrates with glutamate as the P-1 amino acid. When
25 these single mutations are combined, the resulting
multiple enzyme mutants perform better than either
precursor. See Example 9.

30 The fifth category of multiple mutants contain the
substitution of up to four amino acids of the B. amyloliquefaciens subtilisin sequence. These mutants
have specific properties which are virtually identical
to the properties of the subtilisin from B. licheniformis. The subtilisin from B. licheniformis
differs from B. amyloliquefaciens subtilisin at 87 out
35 of 275 amino acids. The multiple mutant

F50/S156/A169/L217 was found to have similar substrate specificity and kinetics to the *licheniformis* enzyme. (See Example 13.) However, this is probably due to only three of the mutations (S156, A169 and L217) which are present in the substrate binding region of the enzyme. It is quite surprising that, by making only three changes out of the 87 different amino acids between the sequence of the two enzymes, the *B. amyloliquifaciens* enzyme was converted into an enzyme with properties similar to *B. licheniformis* enzyme. Other enzymes in this series include F50/Q156/N166/L217 and F50/S156/L217.

The sixth category of multiple mutants includes the combination of substitutions at position 107 (Ile to V) with the substitution of Lys at position 213 with Arg, and the combination of substitutions of position 204 (preferably Ser to C or L but also to all other amino acids) with the substitution of Lys at position 213 with R. Other multiple mutants which have altered alkaline stability include Q156/K166, Q156/N166, S156/K166, S156/N166 (previously identified as having altered substrate specificity), and F50/S156/A169/L217 (previously identified as a mutant of *B. amyloliquifaciens* subtilisin having properties similar to subtilisin from *B. licheniformis*). The mutant F50/V107/R213 was constructed based on the observed increase in alkaline stability for the single mutants F50, V107 and R213. It was determined that the V107/R213 mutant had an increased alkaline stability as compared to the wild type subtilisin. In this particular mutant, the increased alkaline stability was the result of the cumulative stability of each of the individual mutations. Similarly, the mutant F50/V107/R213 had an even greater alkaline stability as compared to the V107/R213 mutant indicating that

the increase in the alkaline stability due to the F50 mutation was also cumulative.

Table IV summarizes the multiple mutants which have been made including those not mentioned above.

5

In addition, based in part on the above results, substitution at the following residues in subtilisin is expected to produce a multiple mutant having increased thermal and alkaline stability: Ser24, Met50, Ile107, Glu156, Gly166, Gly169, Ser204, Lys213, Gly215, and Tyr217.

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TABLE IV

	<u>Double Mutants</u>	<u>Triple, Quadruple or Other Multiple</u>
	C22/C87	F50/I124/Q222
	C24/C87	F50/L124/Q222
5	V45/V48	F50/L124/A222
	C49/C94	A21/C22/C87
	C49/C95	F50/S156/N166/L217
	C50/C95	F50/Q156/N166/L217
	C50/C110	F50/S156/A169/L217
10	F50/I124	F50/S156/L217
	F50/Q222	F50/Q156/K166/L217
	I124/Q222	F50/S156/K166/L217
	Q156/D166	F50/Q156/K166/K217
	Q156/K166	F50/S156/K166/K217
15	Q156/N166	F50/V107/R213
	S156/D166	[S153/S156/A158/G159/S160/A161- 164/I165/S166/A169/R170]
	S156/K166	L204/R213
	S156/N166	R213/204A, E, Q, D, N, G, K, V, R, T, P, I, M, F, Y, W or H
	S156/A169	V107/R213
20	A166/A222	
	A166/C222	
	F166/A222	
	F166/C222	
	K166/A222	
25	K166/C222	
	V166/A222	
	V166/C222	
	A169/A222	
	A169/A222	
30	A169/C222	
	A21/C22	

In addition to the above identified amino acid
residues, other amino acid residues of subtilisin are

also considered to be important with regard to substrate specificity. Mutation of each of these residues is expected to produce changes in the substrate specificity of subtilisin. Moreover, multiple mutations among these residues and among the previously identified residues are also expected to produce subtilisin mutants having novel substrate specificity.

Particularly important residues are His67, Ile107, Leu126 and Leu135. Mutation of His67 should alter the S-1' subsite, thereby altering the specificity of the mutant for the P-1' substrate residue. Changes at this position could also affect the pH activity profile of the mutant. This residue was identified based on the inventor's substrate modeling from product inhibitor complexes.

Ile107 is involved in P-4 binding. Mutation at this position thus should alter specificity for the P-4 substrate residue in addition to the observed effect on alkaline stability. Ile107 was also identified by molecular modeling from product inhibitor complexes.

The S-2 binding site includes the Leu126 residue. Modification at this position should therefore affect P-2 specificity. Moreover, this residue is believed to be important to convert subtilisin to an amino peptidase. The pH activity profile should also be modified by appropriate substitution. These residues were identified from inspection of the refined model, the three dimensional structure from modeling studies. A longer side chain is expected to preclude binding of any side chain at the S-2 subsite. Therefore, binding would be restricted to subsites S-1, S-1', S-2', S-3'

and cleavage would be forced to occur after the amino terminal peptide.

Leu135 is in the S-4 subsite and if mutated should alter substrate specificity for P-4 if mutated. This residue was identified by inspection of the three-dimensional structure and modeling based on the product inhibitor complex of F222.

In addition to these sites, specific amino acid residues within the segments 97-103, 126-129 and 213-215 are also believed to be important to substrate binding.

Segments 97-103 and 126-129 form an antiparallel beta sheet with the main chain of substrate residues P-4 through P-2. Mutating residues in those regions should affect the substrate orientation through main chain (enzyme) - main chain (substrate) interactions, since the main chain of these substrate residues do not interact with these particular residues within the S-4 through S-2 subsites.

Within the segment 97-103, Gly97 and Asp99 may be mutated to alter the position of residues 101-103 within the segment. Changes at these sites must be compatible, however. In B. amyloliquefaciens subtilisin Asp99 stabilizes a turn in the main chain tertiary folding that affects the direction of residues 101-103. B. licheniformis subtilisin Asp97, functions in an analogous manner.

In addition to Gly97 and Asp99, Ser101 interacts with Asp99 in B. amyloliquefaciens subtilisin to stabilize the same main chain turn. Alterations at this residue should alter the 101-103 main chain direction.

Mutations at Glu103 are also expected to affect the 101-103 main chain direction.

5 The side chain of Gly102 interacts with the substrate P-3 amino acid. Side chains of substituted amino acids thus are expected to significantly affect specificity for the P-3 substrate amino acids.

10 All the amino acids within the 127-129 segment are considered important to substrate specificity. Gly 127 is positioned such that its side chain interacts with the S-1 and S-3 subsites. Altering this residue thus should alter the specificity for P-1 and P-3 residues of the substrate.

15 The side chain of Gly128 comprises a part of both the S-2 and S-4 subsites. Altered specificity for P-2 and P-4 therefore would be expected upon mutation. Moreover, such mutation may convert subtilisin into an amino peptidase for the same reasons substitutions of
20 Leu126 would be expected to produce that result.

25 The Pro129 residue is likely to restrict the conformational freedom of the sequence 126-133, residues which may play a major role in determining P-1 specificity. Replacing Pro may introduce more flexibility thereby broadening the range of binding capabilities of such mutants.

30 The side chain of Lys213 is located within the S-3 subsite. All of the amino acids within the 213-215 segment are also considered to be important to substrate specificity. Accordingly, altered P-3 substrate specificity is expected upon mutation of this residue.

The Tyr214 residue does not interact with substrate but is positioned such that it could affect the conformation of the hair pin loop 204-217.

5 Finally, mutation of the Gly215 residue should affect the S-3' subsite, and thereby alter P-3' specificity.

10 In addition to the above substitutions of amino acids, the insertion or deletion of one or more amino acids within the external loop comprising residues 152-172 may also affect specificity. This is because these residues may play a role in the "secondary contact region" described in the model of streptomyces subtilisin inhibitor complexed with subtilisin.

15 Hirono, et al. (1984) J. Mol. Biol. 178, 389-413. Thermitase K has a deletion in this region, which eliminates several of these "secondary contact" residues. In particular, deletion of residues 161 through 164 is expected to produce a mutant subtilisin having modified substrate specificity. In addition, a rearrangement in this area induced by the deletion should alter the position of many residues involved in substrate binding, predominantly at P-1. This, in turn, should affect overall activity against

25 The effect of deletion of residues 161 through 164 has been shown by comparing the activity of the wild type (WT) enzyme with a mutant enzyme containing this deletion as well as multiple substitutions (i.e., S153/S156/A158/G159/S160/ Δ 161-164/I165/S166/A169/R170). This produced the following results:

TABLE V

	<u>kcat</u>	<u>Km</u>	<u>kcat/Km</u>
WT	50	1.4×10^{-4}	3.6×10^5
Deletion mutant	8	5.0×10^{-6}	1.6×10^6

5

The WT has a kcat 6 times greater than the deletion mutant but substrate binding is 28 fold tighter by the deletion mutant. The overall efficiency of the deletion mutant is thus 4.4 times higher than the WT enzyme.

10

All of these above identified residues which have yet to be substituted, deleted or inserted into are presented in Table VI.

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TABLE VI

Substitution/Insertion/Deletion

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Residues

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His67	Ala152
Leu126	Ala153
Leu135	Gly154
Gly97	Asn155
Asp99	Gly156
Ser101	Gly157
Gly102	Gly160
Glu103	Thr158
Leu126	Ser159
Gly127	Ser161
Gly128	Ser162
Pro129	Ser163
Tyr214	Thr164
Gly215	Val165
Gly166	Gly169
Tyr167	Lys170
Pro168	Tyr171
	Pro172

The following disclosure is intended to serve as a representation of embodiments herein, and should not be construed as limiting the scope of this application. These specific examples disclose the construction of certain of the above identified mutants. The construction of the other mutants, however, is apparent from the disclosure herein and that presented in EPO Publication No. 0130756.

All literature citations are expressly incorporated by reference.

EXAMPLE 1

Identification of Peracid Oxidizable Residues of Subtilisin Q222 and L222

As shown in Figures 6A and 6B, organic peracid oxidants inactivate the mutant subtilisins Met222L and Met222Q (L222 and Q222). This example describes the identification of peracid oxidizable sites in these mutant subtilisins.

First, the type of amino acid involved in peracid oxidation was determined. Except under drastic conditions (Means, G.E., et al. (1971) Chemical Modifications of Proteins, Holden-Day, S.F., CA, pp. 160-162), organic peracids modify only methionine and tryptophan in subtilisin. Difference spectra of the enzyme over the 250nm to 350nm range were determined during an inactivation titration employing the reagent, diperdodecanoic acid (DPDA) as oxidant. Despite quantitative inactivation of the enzyme, no change in absorbance over this wavelength range was noted as shown in Figures 7A and 7B indicating that tryptophan was not oxidized. Fontana, A., et al. (1980) Methods in Peptide and Protein Sequence

Analysis (C. Birr ed.) Elsevier, New York, p. 309. The absence of tryptophan modification implied oxidation of one or more of the remaining methionines of B. amyloliquefaciens subtilisin. See Figure 1.

5 To confirm this result the recombinant subtilisin Met222F was cleaved with cyanogen bromide (CNBr) both before and after oxidation by DPDA. The peptides produced by CNBr cleavage were analyzed on high resolution SDS-pyridine peptide gels (SPG).

10 Subtilisin Met222F (F222) was oxidized in the following manner. Purified F222 was resuspended in 0.1 M sodium borate pH 9.5 at 10 mg/ml and was added to a final concentration of 26 dipeperdodecanoic acid (DPDA) at 26 mg/ml was added to produce an effective active oxygen concentration of 30 ppm. The sample was incubated for at least 30 minutes at room temperature and then quenched with 0.1 volume of 1 M Tris pH 8.6 buffer to produce a final concentration of 0.1 M Tris pH 8.6). 3mM phenylmethysulfonyl fluoride (PMSF) was added and 2.5 ml of the sample was applied to a Pharmacia PD10 column equilibrated in 10 mM sodium phosphate pH 6.2, 1 mM PMSF. 3.5 ml of 10 mM sodium phosphate pH6.2, 1mM PMSF was applied and the eluant collected.

25 F222 and DPDA oxidized F222 were precipitated with 9 volumes of acetone at -20°C. The samples were resuspended at 10 mg/ml in 8M urea in 88% formic acid and allowed to sit for 5 minutes. An equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein) and the samples incubated for 2 hours at room temperature in the dark. Prior to gel electrophoresis, the samples were lyophilized and resuspended at 2-5 mg/ml in sample buffer (1%

pyridine, 5% NaDodSO₄, 5% glycerol and bromophenol blue) and disassociated at 95°C for 3 minutes.

The samples were electrophoresed on discontinuous polyacrylamide gels (Kyte, J., et al. (1983) Anal. Bioch. 133, 515-522). The gels were stained using the Pharmacia silver staining technique (Sammons, D.W., et al. (1981) Electrophoresis 2 135-141).

10 The results of this experiment are shown in Figure 8. As can be seen, F222 treated with CNBr only gives nine resolved bands on SPG. However, when F222 is also treated with DPDA prior to cleavage, bands X, 7 and 9 disappear whereas bands 5 and 6 are greatly increased in intensity.

20 In order to determine which of the methionines were effected, each of the CNBr peptides was isolated by reversed phase HPLC and further characterized. The buffer system in both Solvent A (aqueous) and Solvent B (organic) for all HPLC separations was 0.05% triethylamine/trifluoroacetic acid (TEA-TFA). In all cases unless noted, solvent A consisted of 0.05% TEA-TFA in H₂O, solvent B was 0.05% TEA-TFA in 1-propanol, and the flow rate was 0.5 ml/minute.

30 For HPLC analysis, two injections of 1 mg enzyme digest were used. Three samples were acetone precipitated, washed and dried. The dried 1 mg samples were resuspended at 10 mg/ml in 8M urea, 88% formic acid; an equal volume of 200 mg/ml CNBr in 88% formic acid was added (5 mg/ml protein). After incubation for 2 hours in the dark at room temperature, the samples were desalted on a 0.8 cm X 7

cm column of Tris Acryl GF05 coarse resin (IBF, Paris, France) equilibrated with 40% solvent B, 60% solvent A. 200 ul samples were applied at a flow rate of 1 ml a minute and 1.0-1.2 ml collected by monitoring the absorbance at 280nm. Prior to injection on the HPLC, each desalted sample was diluted with 3 volumes of solvent A. The samples were injected at 1.0 ml/min (2 minutes) and the flow then adjusted to 0.5 ml/min (100% A). After 2 minutes, a linear gradient to 60% B at 1.0% B/min was initiated. From each 1 mg run, the pooled peaks were sampled (50ul) and analyzed by gel electrophoresis as described above.

Each polypeptide isolated by reversed phase HPLC was further analyzed for homogeneity by SPG. The position of each peptide on the known gene sequence (Wells, J.A., et al. (1983) Nucleic Acids Res. 11 7911-7924) was obtained through a combination of amino acid compositional analysis and, where needed, amino terminal sequencing.

Prior to such analysis the following peptides were to rechromatographed.

1. CNBr peptides from F222 not treated with DPDA:

Peptide 5 was subjected to two additional reversed phase separations. The 10 cm C4 column was equilibrated to 80%A/ 20%B and the pooled sample applied and washed for 2 minutes. Next an 0.5% ml B/min gradient was initiated. Fractions from this separation were again rerun, this time on the 25 cm C4 column, and employing 0.05% TEA-TFA in acetonitrile/1-propanol (1:1) for solvent B. The gradient was identical to the one just described.

Peptide "X" was subjected to one additional separation after the initial chromatography. The sample was applied and washed for 2 minutes at 0.5ml/min (100% A), and a 0.5% ml B/min gradient was initiated.

- 5 Peptides 7 and 9 were rechromatographed in a similar manner to the first rerun of peptide 5.

Peptide 8 was purified to homogeneity after the initial separation.

10

2. CNBr Peptides from DPDA Oxidized F222:

- 15 Peptides 5 and 6 from a CNBr digest of the oxidized F222 were purified in the same manner as peptide 5 from the untreated enzyme.

20 Amino acid compositional analysis was obtained as follows. Samples (~1nM each amino acid) were dried, hydrolyzed in vacuo with 100 ul 6N HCl at 106°C for 24 hours and then dried in a Speed Vac. The samples were analyzed on a Beckmann 6300 AA analyzer employing ninhydrin detection.

25 Amino terminal sequence data was obtained as previously described (Rodriguez, H., et al. (1984) Anal. Biochem. 134, 538-547).

The results are shown in Table VII and Figure 9.

30

35

TABLE VII

Amino and COOH termini of CNBr fragments

		<u>Terminus and Method</u>	
	<u>Fragment</u>	<u>amino, method</u>	<u>COOH, method</u>
5	X	1, sequence	50, composition
	9	51, sequence	119, composition
	7	125, sequence	199, composition
	8		
10		200, sequence	275, composition
	5ox	1, sequence	119, composition
	6ox	120, composition	199, composition

15 Peptides 5ox and 6ox refer to peptides 5 and 6 isolated from CNBr digests of the oxidized protein where their respective levels are enhanced.

20 From the data in Table VII and the comparison of SPG tracks for the oxidized and native protein digests in Figure 8, it is apparent that (1) Met50 is oxidized leading to the loss of peptides X and 9 and the appearance of 5; and (2) Met124 is also oxidized leading to the loss of peptide 7 and the accumulation of peptide 6. Thus oxidation of B. amyloliquifaciens subtilisin with the peracid, diperdocecanoic acid leads to the specific oxidation of methionine at residues 50 and 124.

30 EXAMPLE 2

Substitution at Met50 and Met124
in Subtilisin Met2220

35 The choice of amino acid for substitution at Met50 was based on the available sequence data for subtilisins

from B. licheniformis (Smith, E.C., et al. (1968) J. Biol. Chem. 243, 2184-2191), B.DY (Nedkov, P., et al. (1983) Hoppe Sayler's Z. Physiol. Chem. 364 1537-1540), B. amylosacchariticus (Markland, F.S., et al. (1967) J. Biol. Chem. 242 5198-5211) and B. subtilis (Stahl, M.L., et al. (1984) J. Bacteriol. 158, 411-418). In all cases, position 50 is a phenylalanine. See Figure 5. Therefore, Phe50 was chosen for construction.

At position 124, all known subtilisins possess a methionine. See Figure 5. Molecular modelling of the x-ray derived protein structure was therefore required to determine the most probable candidates for substitution. From all 19 candidates, isoleucine and leucine were chosen as the best residues to employ. In order to test whether or not modification at one site but not both was sufficient to increase oxidative stability, all possible combinations were built on the Q222 backbone (F50/Q222, I124/Q222, F50/I124/Q222).

A. Construction of Mutations Between Codons 45 and 50

All manipulations for cassette mutagenesis were carried out on pS4.5 using methods disclosed in EPO Publication No. 0130756 and Wells, J.A., et al, (1985) Gene 34, 315-323. The pΔ50 in Fig. 10, line 4, mutations was produced using the mutagenesis primer shown in Fig. 10, line 6, and employed an approach designated as restriction-purification which is described below. Briefly, a M13 template containing the subtilisin gene, M13mpl1-SUBT was used for heteroduplex synthesis (Adelman, et al (1983), DNA 2, 183-193). Following transfection of JM101 (ATCC 33876), the 1.5 kb EcoRI-BamHI fragment containing the

subtilisin gene was subcloned from M13mp11 SUBT rf into a recipient vector fragment of pBS42 the construction of which is described in EPO Publication No. 0130756. To enrich for the mutant sequence (pΔ50, line 4), the resulting plasmid pool was digested with KpnI, and linear molecules were purified by polyacrylamide gel electrophoresis. Linear molecules were ligated back to a circular form, and transformed into E. coli MM294 cells (ATCC 31446). Isolated plasmids were screened by restriction analysis for the KpnI site. KpnI⁺ plasmids were sequenced and confirmed the pΔ50 sequence. Asterisks in Figure 11 indicate the bases that are mutated from the wild type sequence (line 4). pΔ50 (line 4) was cut with StuI and EcoRI and the 0.5 Kb fragment containing the 5' half of the subtilisin gene was purified (fragment 1). pΔ50 (line 4) was digested with KpnI and EcoRI and the 4.0 Kb fragment containing the 3' half of the subtilisin gene and vector sequences was purified (fragment 2). Fragments 1 and 2 (line 5), and duplex DNA cassettes coding for mutations desired (shaded sequence, line 6) were mixed in a molar ratio of 1:1:10, respectively. For the particular construction of this example the DNA cassette contained the triplet TTT for codon 50 which encodes Phe. This plasmid was designated pF50. The mutant subtilisin was designated F50.

B. Construction of Mutation
Between Codons 122 and 127

The procedure of Example 2A was followed in substantial detail except that the mutagenesis primer of Figure 11, line 7 was used and restriction-purification for the EcoRV site in pΔ124 was used. In addition, the DNA cassette (shaded sequence, Figure

11, line 6) contained the triplet ATT for codon 124 which encodes Ile and CTT for Leu. Those plasmids which contained the substitution of Ile for Met124 were designated pI124. The mutant subtilisin was designated I124.

5

C. Construction of Various
F50/I124/Q222 Multiple Mutants

10 The triple mutant, F50/I124/Q222, was constructed from a three-way ligation in which each fragment contained one of the three mutations. The single mutant Q222 (pQ222) was prepared by cassette mutagenesis as described in EPO Publication No. 0130756. The F50 mutation was contained on a 2.2kb AvaII to PvuII fragment from pF50; the I124 mutation was contained on a 260 bp PvuII to AvaII fragment from pI124; and the Q222 mutation was contained on 2.7 kb AvaII to AvaII fragment from pQ222. The three fragments were ligated together and transformed into E. coli MM294 cells.

15

20 Restriction analysis of plasmids from isolated transformants confirmed the construction. To analyze the final construction it was convenient that the AvaII site at position 798 in the wild-type subtilisin gene was eliminated by the I124 construction.

25

The F50/Q222 and I124/Q222 mutants were constructed in a similar manner except that the appropriate fragment from pS4.5 was used for the final construction.

30

D. Oxidative Stability of Q222 Mutants

The above mutants were analyzed for stability to peracid oxidation. As shown in Fig. 12, upon incubation with diperdodecanoic acid (protein 2mg/mL, oxidant 75ppm[O]), both the I124/Q222 and the

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F50/I124/Q222 are completely stable whereas the F50/Q222 and the Q222 are inactivated. This indicates that conversion of Met124 to I124 in subtilisin Q222 is sufficient to confer resistance to organic peracid oxidants.

5

EXAMPLE 3

10 Subtilisin Mutants Having Altered Substrate Specificity-Hydrophobic Substitutions at Residues 166

Subtilisin contains an extended binding cleft which is hydrophobic in character. A conserved glycine at residue 166 was replaced with twelve non-ionic amino acids which can project their side-chains into the S-1 subsite. These mutants were constructed to determine the effect of changes in size and hydrophobicity on the binding of various substrates.

20

A. Kinetics for Hydrolysis of Substrates Having Altered P-1 Amino Acids by Subtilisin from B. Amylolyquefaciens

Wild-type subtilisin was purified from B. subtilis culture supernatants expressing the B. amylolyquefaciens subtilisin gene (Wells, J.A., et al. (1983) Nucleic Acids Res. 11, 7911-7925) as previously described (Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521). Details of the synthesis of tetrapeptide substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide (where X is the P1 amino acid) are described by DelMar, E.G., et al. (1979) Anal. Biochem. 99, 316-320. Kinetic parameters, $K_m(M)$ and $k_{cat}(s^{-1})$ were measured using a modified progress curve analysis (Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521). Briefly, plots

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of rate versus product concentration were fit to the differential form of the rate equation using a non-linear regression algorithm. Errors in k_{cat} and K_m for all values reported are less than five percent. The various substrates in Table VIII are ranged in order of decreasing hydrophobicity. Nozaki, Y. (1971), J. Biol. Chem. 246, 2211-2217; Tanford C. (1978) Science 200, 1012).

10

TABLE VIII

	P1 substrate Amino Acid	$k_{cat}(S^{-1})$	$1/K_m(M^{-1})$	k_{cat}/K_m ($S^{-1}M^{-1}$)
15	Phe	50	7,100	360,000
	Tyr	28	40,000	1,100,000
	Leu	24	3,100	75,000
	Met	13	9,400	120,000
	His	7.9	1,600	13,000
20	Ala	1.9	5,500	11,000
	Gly	0.003	8,300	21
	Gln	3.2	2,200	7,100
	Ser	2.8	1,500	4,200
	Glu	0.54	32	16

25

The ratio of k_{cat}/K_m (also referred to as catalytic efficiency) is the apparent second order rate constant for the conversion of free enzyme plus substrate (E+S) to enzyme plus products (E+P) (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287). The log (k_{cat}/K_m) is proportional to transition state binding

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energy, ΔG_T^\ddagger . A plot of the $\log k_{cat}/K_m$ versus the hydrophobicity of the P1 side-chain (Figure 14) shows a strong correlation ($r = 0.98$), with the exception of the glycine substrate which shows evidence for non-productive binding. These data show that relative differences between transition-state binding energies can be accounted for by differences in P-1 side-chain hydrophobicity. When the transition-state binding energies are calculated for these substrates and plotted versus their respective side-chain hydrophobicities, the line slope is 1.2 (not shown). A slope greater than unity, as is also the case for chymotrypsin (Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Harper, J.W., et al. (1984) Biochemistry, 23, 2995-3002), suggests that the P1 binding cleft is more hydrophobic than ethanol or dioxane solvents that were used to empirically determine the hydrophobicity of amino acids (Nozaki, Y., et al. J. Biol. Chem. (1971) 246, 2211-2217; Tanford, C. (1978) Science 200, 1012).

For amide hydrolysis by subtilisin, k_{cat} can be interpreted as the acylation rate constant and K_m as the dissociation constant, for the Michaelis complex (E-S), Ks. Gutfreund, H., et al (1956) Biochem. J. 63, 656. The fact that the $\log k_{cat}$, as well as $\log 1/K_m$, correlates with substrate hydrophobicity is consistent with proposals (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303) that during the acylation step the P-1 side-chain moves deeper into the hydrophobic cleft as the substrate advances from the Michaelis complex (E-S) to the tetrahedral transition-state complex (E-S ‡). However, these data can also be interpreted as the hydrophobicity of the P1 side-chain effecting the orientation, and thus the

susceptibility of the scissile peptide bond to nucleophilic attack by the hydroxyl group of the catalytic Ser221.

5 The dependence of k_{cat}/K_m on P-1 side chain hydrophobicity suggested that the k_{cat}/K_m for hydrophobic substrates may be increased by increasing the hydrophobicity of the S-1 binding subsite. To test this hypothesis, hydrophobic amino acid
10 substitutions of Gly166 were produced.

Since hydrophobicity of aliphatic side-chains is directly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927), increasing the hydrophobicity in
15 the S-1 subsite may also sterically hinder binding of larger substrates. Because of difficulties in predicting the relative importance of these two opposing effects, we elected to generate twelve
20 non-charged mutations at position 166 to determine the resulting specificities against non-charged substrates of varied size and hydrophobicity.

25 B. Cassette Mutagenesis of the P1 Binding Cleft

The preparation of mutant subtilisins containing the substitution of the hydrophobic amino acids Ala, Val and Phe into residue 166 has been described in EPO
30 Publication No. 0130756. The same method was used to produce the remaining hydrophobic mutants at residue 166. In applying this method, two unique and silent restriction sites were introduced in the subtilisin genes to closely flank the target codon 166. As can
35 be seen in Figure 13, the wild type sequence (line 1)

was altered by site-directed mutagenesis in M13 using the indicated 37mer mutagenesis primer, to introduce a 13 bp deletion (dashedline) and unique SacI and XmaI sites (underlined sequences) that closely flank codon 166. The subtilisin gene fragment was subcloned back into the E. coli - B. subtilis shuttle plasmid, pBS42, giving the plasmid pA166 (Figure 13, line 2). pA166 was cut open with SacI and XmaI, and gapped linear molecules were purified (Figure 13, line 3). Pools of synthetic oligonucleotides containing the mutation of interest were annealed to give duplex DNA cassettes that were ligated into gapped pA166 (underlined and overlined sequences in Figure 13, line 4). This construction restored the coding sequence except over position 166(NNN; line 4). Mutant sequences were confirmed by dideoxy sequencing. Asterisks denote sequence changes from the wild type sequence. Plasmids containing each mutant B. amyloliquefaciens subtilisin gene were expressed at roughly equivalent levels in a protease deficient strain of B. subtilis, BG2036 as previously described. EPO Publication No. 0130756; Yang, M., et al. (1984) J. Bacteriol. 160, 15-21; Estell, D.A., et al. (1985) J. Biol. Chem. 260, 6518-6521.

C. Narrowing Substrate Specificity by Steric Hindrance

To probe the change in substrate specificity caused by steric alterations in the S-1 subsite, position 166 mutants were kinetically analyzed versus P1 substrates of increasing size (i.e., Ala, Met, Phe and Tyr). Ratios of k_{cat}/K_m are presented in log form in Figure 15 to allow direct comparisons of transition-state binding energies between various enzyme-substrate pairs.

According to transition state theory, the free energy difference between the free enzyme plus substrate (E + S) and the transition state complex (E.S[‡]) can be calculated from equation (1),

5 (1) $\Delta G_T^\ddagger = -RT \ln k_{cat}/K_m + RT \ln kT/h$

in which k_{cat} is the turnover number, K_m is the Michaelis constant, R is the gas constant, T is the temperature, k is Boltzmann's constant, and h is Planck's constant. Specificity differences are expressed quantitatively as differences between transition state binding energies (i.e., $\Delta\Delta G_t^\ddagger$), and can be calculated from equation (2).

15 (2) $\Delta\Delta G_T^\ddagger = -RT \ln (k_{cat}/K_m)_A / (k_{cat}/K_m)_B$

A and B represent either two different substrates assayed against the same enzyme, or two mutant enzymes assayed against the same substrate.

20 As can be seen from Figure 15A, as the size of the side-chain at position 166 increases the substrate preference shifts from large to small P-1 side-chains. Enlarging the side-chain at position 166 causes k_{cat}/K_m to decrease in proportion to the size of the P-1 substrate side-chain (e.g., from Gly166 (wild-type) through W166, the k_{cat}/K_m for the Tyr substrate is decreased most followed in order by the Phe, Met and Ala P-1 substrates).

30 Specific steric changes in the position 166 side-chain, such as the presence of a β -hydroxyl group, β - or γ -aliphatic branching, cause large decreases in k_{cat}/K_m for larger P1 substrates. Introducing a β -hydroxyl group in going from A166 (Figure 15A) to

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S166 (Figure 15B), causes an 8 fold and 4 fold reduction in k_{cat}/K_m for Phe and Tyr substrates, respectively, while the values for Ala and Met substrates are unchanged. Producing a β -branched structure, in going from S166 to T166, results in a drop of 14 and 4 fold in k_{cat}/K_m for Phe and Tyr, respectively. These differences are slightly magnified for V166 which is slightly larger and isosteric with T166. Enlarging the β -branched substituents from V166 to I166 causes a lowering of k_{cat}/K_m between two and six fold toward Met, Phe and Tyr substrates. Inserting a γ -branched structure, by replacing M166 (Figure 15A) with L166 (Figure 15B), produces a 5 fold and 18 fold decrease in k_{cat}/K_m for Phe and Tyr substrates, respectively. Aliphatic γ -branched appears to induce less steric hindrance toward the Phe P-1 substrate than β -branching, as evidenced by the 100 fold decrease in k_{cat}/K_m for the Phe substrate in going from L166 to I166.

Reductions in k_{cat}/K_m resulting from increases in side chain size in the S-1 subsite, or specific structural features such as β - and γ -branching, are quantitatively illustrated in Figure 16. The k_{cat}/K_m values for the position 166 mutants determined for the Ala, Met, Phe, and Tyr P-1 substrates (top panel through bottom panel, respectively), are plotted versus the position 166 side-chain volumes (Chothia, C. (1984) Ann. Rev. Biochem. 53, 537-572). Catalytic efficiency for the Ala substrate reaches a maximum for I166, and for the Met substrate it reaches a maximum between V166 and L166. The Phe substrate shows a broad k_{cat}/K_m peak but is optimal with A166. Here, the β -branched position 166 substitutions form a line that is parallel to, but roughly 50 fold lower in k_{cat}/K_m than side-chains of similar size [i.e., C166 versus

Tl66, Ll66 versus Il66]. The Tyr substrate is most efficiently utilized by wild type enzyme (Glyl66), and there is a steady decrease as one proceeds to large position 166 side-chains. The β -branched and γ -branched substitutions form a parallel line below the other non-charged substitutions of similar molecular volume.

The optimal substitution at position 166 decreases in volume with increasing volume of the P1 substrate [i.e., Il66/Ala substrate, Ll66/Met substrate, Al66/Phe substrate, Glyl66/Tyr substrate]. The combined volumes for these optimal pairs may approximate the volume for productive binding in the S-1 subsite. For the optimal pairs, Glyl66/Tyr substrate, Al66/Phe substrate, Ll66/Met substrate, Vl66/Met substrate, and Il66/Ala substrate, the combined volumes are 266, 295, 313, 339 and 261 \AA^3 , respectively. Subtracting the volume of the peptide backbone from each pair (i.e., two times the volume of glycine), an average side-chain volume of $160 \pm 32 \text{\AA}^3$ for productive binding can be calculated.

The effect of volume, in excess to the productive binding volume, on the drop in transition-state binding energy can be estimated from the Tyr substrate curve (bottom panel, Figure 16), because these data, and modeling studies (Figure 2), suggest that any substitution beyond glycine causes steric repulsion. A best-fit line drawn to all the data ($r = 0.87$) gives a slope indicating a loss of roughly 3 kcal/mol in transition state binding energy per 100\AA^3 of excess volume. (100\AA^3 is approximately the size of a leucyl side-chain.)

D. Enhanced Catalytic Efficiency
Correlates with Increasing Hydrophobicity
of the Position 166 Substitution

Substantial increases in k_{cat}/K_m occur with enlargement of the position 166 side-chain, except for the Tyr P-1 substrate (Figure 16). For example, k_{cat}/K_m increases in progressing from Gly166 to Ile166 for the Ala substrate (net of ten-fold), from Gly166 to Leu166 for the Met substrate (net of ten-fold) and from Gly166 to Ala166 for the Phe substrate (net of two-fold). The increases in k_{cat}/K_m cannot be entirely explained by the attractive terms in the van der Waals potential energy function because of their strong distance dependence ($1/r^6$) and because of the weak nature of these attractive forces (Jencks, W.P., Catalysis in Chemistry and Enzymology (McGraw-Hill, 1969) pp. 321-436; Fersht, A., Enzyme Structure and Mechanism (Freeman, San Francisco, 1977) pp. 226-287; Levitt, M. (1976) J. Mol. Biol. 104, 59-107). For example, Levitt (Levitt, M. (1976) J. Mol. Biol. 104, 59-107) has calculated that the van der Waals attraction between two methionyl residues would produce a maximal interaction energy of roughly -0.2 kcal/mol. This energy would translate to only 1.4 fold increase in k_{cat}/K_m .

The increases of catalytic efficiency caused by side-chain substitutions at position 166 are better accounted for by increases in the hydrophobicity of the S-1 subsite. The increase k_{cat}/K_m observed for the Ala and Met substrates with increasing position 166 side-chain size would be expected, because hydrophobicity is roughly proportional to side-chain surface area (Rose, G.D., et al. (1985) Science 229, 834-838; Reynolds, J.A., et al. (1974) Proc. Natl. Acad. Sci. USA 71, 2825-2927).

Another example that can be interpreted as a hydrophobic effect is seen when comparing k_{cat}/K_m for isosteric substitutions that differ in hydrophobicity such as S166 and C166 (Figure 16). Cysteine is considerably more hydrophobic than serine (-1.0 versus +0.3 kcal/mol) (Nozaki, Y., et al. (1971) J. Biol. Chem. 246, 2211-2217; Tanford, C. (1978) Science 200, 1012). The difference in hydrophobicity correlates with the observation that C166 becomes more efficient relative to Ser166 as the hydrophobicity of the substrates increases (i.e., Ala < Met < Tye < Phe). Steric hindrance cannot explain these differences because serine is considerably smaller than cysteine (99 versus 118 Å³). Paul, I.C., Chemistry of the -SH Group (ed. S. Patai, Wiley Interscience, New York, 1974) pp. 111-149.

E. Production of an Elastase-Like Specificity in Subtilisin

The I166 mutation illustrates particularly well that large changes in specificity can be produced by altering the structure and hydrophobicity of the S-1 subsite by a single mutation (Figure 17). Progressing through the small hydrophobic substrates, a maximal specificity improvement over wild type occurs for the Val substrate (16 fold in k_{cat}/K_m). As the substrate side chain size increases, these enhancements shrink to near unity (i.e., Leu and His substrates). The I166 enzyme becomes poorer against larger aromatic substrates of increasing size (e.g., I166 is over 1,000 fold worse against the Tyr substrate than is Gly166). We interpret the increase in catalytic efficiency toward the small hydrophobic substrates for I166 compared to Gly166 to the greater hydrophobicity of isoleucine (i.e., -1.8 kcal/mol versus 0). Nozaki,

Y., et al. (1971) J. Biol. Chem. 246, 2211-2217;
Tanford, C. (1978) Science 200, 1012. The decrease in
catalytic efficiency toward the very large substrates
for Il66 versus Glyl66 is attributed to steric
repulsion.

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The specificity differences between Glyl66 and Il66
are similar to the specificity differences between
chymotrypsin and the evolutionary relative, elastase
(Harper, J.W., et al. (1984) Biochemistry 23,
10 2995-3002). In elastase, the bulky amino acids, Thr
and Val, block access to the P-1 binding site for
large hydrophobic substrates that are preferred by
chymotrypsin. In addition, the catalytic efficiencies
toward small hydrophobic substrates are greater for
15 elastase than for chymotrypsin as we observe for Il66
versus Glyl66 in subtilisin.

EXAMPLE 4

20 Substitution of Ionic Amino Acids for Glyl66

The construction of subtilisin mutants containing the
substitution of the ionic amino acids Asp, Asn, Gln,
Lys and Arg are disclosed in EPO Publication No.
25 0130756. The present example describes the
construction of the mutant subtilisin containing Glu
at position 166 (El66) and presents substrate
specificity data on these mutants. Further data on
position 166 and 156 single and double mutants is
30 presented infra.

pAl66, described in Example 3, was digested with SacI
and XmaI. The double strand DNA cassette (underlined
and overlined) of line 4 in Figure 13 contained the

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triplet GAA for the codon 166 to encode the replacement of Glu for Gly166. This mutant plasmid designated pQ166 was propagated in BG2036 as described. This mutant subtilisin, together with the other mutants containing ionic substituent amino acids at residue 166, were isolated as described and further analyzed for variations in substrate specificity.

Each of these mutants was analyzed with the tetrapeptide substrates, succinyl-L-AlaL-AlaProL-X-p-nitroanilide, where X was Phe, Ala and Glu.

The results of this analysis are shown in Table IX.

TABLE IX

<u>Position 166</u>	<u>P-1 Substrate</u> (kcat/Km x 10 ⁻⁴)		
	<u>Phe</u>	<u>Ala</u>	<u>Glu</u>
Gly (wild type)	36.0	1.4	0.002
Asp (D)	0.5	0.4	<0.001
Glu (E)	3.5	0.4	<0.001
Asn (N)	18.0	1.2	0.004
Gln (Q)	57.0	2.6	0.002
Lys (K)	52.0	2.8	1.2
Arg (R)	42.0	5.0	0.08

These results indicate that charged amino acid substitutions at Gly166 have improved catalytic efficiencies (kcat/Km) for oppositely charged P-1 substrates (as much as 500 fold) and poorer catalytic efficiency for like charged P-1 substrates.

EXAMPLE 5

Substitution of Glycine at Position 169

The substitution of Gly169 in *B. amyloliquefaciens* subtilisin with Ala and Ser is described in EPO Publication No. 0130756. The same method was used to make the remaining 17 mutants containing all other substituent amino acids for position 169.

The construction protocol is summarized in Figure 18. The overscored and underscored double stranded DNA cassettes used contained the following triplet encoding the substitution of the indicated amino acid at residue 169.

15	GCT	A	ATG	M
	TGT	C	AAC	N
	GAT	D	CCT	P
	GAA	E	CAA	Q
20	TTC	F	AGA	R
	GGC	G	AGC	S
	CAC	H	ACA	T
	ATC	I	GTT	V
	AAA	K	TGG	W
25	CTT	L	TAC	Y

Each of the plasmids containing a substituted Gly169 was designated pX169, where X represents the substituent amino acid. The mutant subtilisins were similarly designated.

Two of the above mutant subtilisins, A169 and S169, were analyzed for substrate specificity against synthetic substrates containing Phe, Leu, Ala and Arg in the P-1 position. The following results are shown in Table X.

TABLE X

Effect of Serine and Alanine Mutations
at Position 169 on P-1 Substrate Specificity

Position 169	P-1 Substrate (kcat/Km x 10 ⁻⁴)			
	<u>Phe</u>	<u>Leu</u>	<u>Ala</u>	<u>Arg</u>
Gly (wild type)	40	10	1	0.4
Al69	120	20	1	0.9
Sl69	50	10	1	0.6

These results indicate that substitutions of Ala and Ser at Gly169 have remarkably similar catalytic efficiencies against a range of P-1 substrates compared to their position 166 counterparts. This is probably because position 169 is at the bottom of the P-1 specificity subsite.

EXAMPLE 6

Substitution at Position 104

Tyr104 has been substituted with Ala, His, Leu, Met and Ser. The method used was a modification of the site directed mutagenesis method. According to the protocol of Figure 19, a primer (shaded in line 4) introduced a unique HindIII site and a frame shift mutation at codon 104. Restriction-purification for the unique HindIII site facilitated the isolation of the mutant sequence (line 4). Restriction-selection against this HindIII site using primers in line 5 was used to obtain position 104 mutants.

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The following triplets were used in the primers of Figure 19, line 5 for the 104 codon which substituted the following amino acids.

5	GCT	A	TTC	F
	ATG	M	CCT	P
	CTT	L	ACA	T
	AGC	S	TGG	W
	CAC	H	TAC	Y
10	CAA	Q	GTT	V
	GAA	E	AGA	R
	GGC	G	AAC	N
	ATC	I	GAT	D
	AAA	K	TGT	C

15 The substrates in Table XI were used to analyze the substrate specificity of these mutants. The results obtained for H104 subtilisin are shown in Table XI.

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TABLE XI

	<u>Substrate</u>	<u>kcat</u>		<u>Km</u>		<u>Kcat/Km</u>	
		<u>WT</u>	<u>H104</u>	<u>WT</u>	<u>H104</u>	<u>WT</u>	<u>H104</u>
25	sAAPFPNA	50.0	22.0	1.4×10^{-4}	7.1×10^{-4}	3.6×10^5	3.1×10^4
	sAAPApNA	3.2	2.0	2.3×10^{-4}	1.9×10^{-3}	1.4×10^4	1×10^3
	sFAPFPNA	26.0	38.0	1.8×10^{-4}	4.1×10^{-4}	1.5×10^5	9.1×10^4
	sFAPApNA	0.32	2.4	7.3×10^{-5}	1.5×10^{-4}	4.4×10^3	1.6×10^4

30

From these data it is clear that the substitution of His for Tyr at position 104 produces an enzyme which is more efficient (higher kcat/Km) when Phe is at the P-4 substrate position than when Ala is at the P-4 substrate position.

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EXAMPLE 7

Substitution of Ala152

Ala152 has been substituted by Gly and Ser to determine the effect of such substitutions on substrate specificity.

The wild type DNA sequence was mutated by the V152/P153 primer (Figure 20, line 4) using the above restriction-purification approach for the new KpnI site. Other mutant primers (shaded sequences Figure 20; S152, line 5 and G152, line 6) mutated the new KpnI site away and such mutants were isolated using the restriction-selection procedure as described above for loss of the KpnI site.

The results of these substitutions for the above synthetic substrates containing the P-1 amino acids Phe, Leu and Ala are shown in Table XII.

TABLE XII

<u>Position 152</u>	<u>P-1 Substrate</u>		
	(kcat/K _m × 10 ⁻⁴)		
	<u>Phe</u>	<u>Leu</u>	<u>Ala</u>
Gly (G)	0.2	0.4	<0.04
Ala (wild type)	40.0	10.0	1.0
Ser (S)	1.0	0.5	0.2

These results indicate that, in contrast to positions 166 and 169, replacement of Ala152 with Ser or Gly causes a dramatic reduction in catalytic efficiencies

across all substrates tested. This suggests Ala152, at the top of the S-1 subsite, may be the optimal amino acid because Ser and Gly are homologous Ala substitutes.

5

EXAMPLE 8

Substitution at Position 156

10 Mutants containing the substitution of Ser and Gln for Glu156 have been constructed according to the overall method depicted in Figure 21. This method was designed to facilitate the construction of multiple mutants at position 156 and 166 as will be described hereinafter. However, by regenerating the wild type
15 Gly166, single mutations at Glu156 were obtained.

20 The plasmid pA166 is already depicted in line 2 of Figure 13. The synthetic oligonucleotides at the top right of Figure 21 represent the same DNA cassettes depicted in line 4 of Figure 13. The plasmid p166 in Figure 21 thus represents the mutant plasmids of Examples 3 and 4. In this particular example, p166 contains the wild type Gly166.

25 Construction of position 156 single mutants were prepared by ligation of the three fragments (1-3) indicated at the bottom of Figure 21. Fragment 3, containing the carboxy-terminal portion of the subtilisin gene including the wild type position 166
30 codon, was isolated as a 610 bp SacI-BamHI fragment. Fragment 1 contained the vector sequences, as well as the amino-terminal sequences of the subtilisin gene through codon 151. To produce fragment 1, a unique KpnI site at codon 152 was introduced into the wild
35 type subtilisin sequence from pS4.5. Site-directed

mutagenesis in M13 employed a primer having the sequence 5'-TA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3' to produce the mutation. Enrichment for the mutant sequence was accomplished by restriction with KpnI, purification and self ligation. The mutant sequence containing the KpnI site was confirmed by direct plasmid sequencing to give pV152. pV152 (~1 µg) was digested with KpnI and treated with 2 units of DNA polymerase I large fragment (Klenow fragment from Boeringer-Mannheim) plus 50 µM deoxynucleotide triphosphates at 37°C for 30 min. This created a blunt end that terminated with codon 151. The DNA was extracted with 1:1 volumes phenol and CHCl₃ and DNA in the aqueous phase was precipitated by addition of 0.1 volumes 5M ammonium acetate and two volumes ethanol. After centrifugation and washing the DNA pellet with 70% ethanol, the DNA was lyophilized. DNA was digested with BamHI and the 4.6kb piece (fragment 1) was purified by acrylamide gel electrophoresis followed by electroelution. Fragment 2 was a duplex synthetic DNA cassette which when ligated with fragments 1 and 3 properly restored the coding sequence except at codon 156. The top strand was synthesized to contain a glutamine codon, and the complementary bottom strand coded for serine at 156. Ligation of heterophosphorylated cassettes leads to a large and favorable bias for the phosphorylated over the non-phosphorylated oligonucleotide sequence in the final segregated plasmid product. Therefore, to obtain Q156 the top strand was phosphorylated, and annealed to the non-phosphorylated bottom strand prior to ligation. Similarly, to obtain S156 the bottom strand was phosphorylated and annealed to the non-phosphorylated top strand. Mutant sequences were isolated after ligation and transformation, and were confirmed by restriction analysis and DNA sequencing

as before. To express variant subtilisins, plasmids were transformed into a subtilisin-neutral protease deletion mutant of *B. subtilis*, BG2036, as previously described. Cultures were fermented in shake flasks for 24 h at 37°C in LB media containing 12.5 mg/mL chloramphenicol and subtilisin was purified from culture supernatants as described. Purity of subtilisin was greater than 95% as judged by SDS PAGE.

These mutant plasmids designated pS156 and pQ156 and mutant subtilisins designated S156 and Q156 were analyzed with the above synthetic substrates where P-1 comprised the amino acids Glu, Gln, Met and Lys. The results of this analyses are presented in Example 9.

EXAMPLE 9

Multiple Mutants With Altered Substrate Specificity - Substitution at Positions 156 and 166

Single substitutions of position 166 are described in Examples 3 and 4. Example 8 describes single substitutions at position 156 as well as the protocol of Figure 21 whereby various double mutants comprising the substitution of various amino acids at positions 156 and 166 can be made. This example describes the construction and substrate specificity of subtilisin containing substitutions at position 156 and 166 and summarizes some of the data for single and double mutants at positions 156 and 166 with various substrates.

K166 is a common replacement amino acid in the 156/166 mutants described herein. The replacement of Lys for

Gly166 was achieved by using the synthetic DNA cassette at the top right of Figure 21 which contained the triplet AAA for NNN. This produced fragment 2 with Lys substituting for Gly166.

5 The 156 substituents were Gln and Ser. The Gln and Ser substitutions at Gly156 are contained within fragment 3 (bottom right Figure 21).

10 The multiple mutants were produced by combining fragments 1, 2 and 3 as described in Example 8. The mutants Q156/K166 and S156/K166 were selectively generated by differential phosphorylation as described. Alternatively, the double 156/166 mutants, c.f. Q156/K166 and S156/K166, were prepared by ligation of the 4.6kb SacI-BamHI fragment from the relevant p156 plasmid containing the 0.6kb SacI-BamHI fragment from the relevant p166 plasmid.

20 These mutants, the single mutant K166, and the S156 and Q156 mutants of Example 8 were analyzed for substitute specificity against synthetic polypeptides containing Phe or Glu as the P-1 substrate residue. The results are presented in Table XIII.

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TABLE XIII

Enzymes Compared (b)	Substrate P-1 Residue	kcat	Km	kcat/Km	
				kcat/Km	kcat/Km (mutant) kcat/Km (wt)
Glu156/Gly166 (WT)	Phe	50.00	1.4×10^{-4}	3.6×10^5	(1)
	Glu	0.54	3.4×10^{-2}	1.6×10^1	(1)
K166	Phe	20.00	4.0×10^{-5}	5.2×10^5	1.4
	Glu	0.70	5.6×10^{-5}	1.2×10^4	750
Q156/K166	Phe	30.00	1.9×10^{-5}	1.6×10^6	4.4
	Glu	1.60	3.1×10^{-5}	5.0×10^4	3100
S156/K166	Phe	30.00	1.8×10^{-5}	1.6×10^6	4.4
	Glu	0.60	3.9×10^{-5}	1.6×10^4	1000
S156	Phe	34.00	4.7×10^{-5}	7.3×10^5	2.0
	Glu	0.40	1.8×10^{-3}	1.1×10^2	6.9
E156	Phe	48.00	4.5×10^{-5}	1.1×10^6	3.1
	Glu	0.90	3.3×10^{-3}	2.7×10^2	17

As can be seen in Table XIV, either of these single mutations improve enzyme performance upon substrates with glutamate at the P-1 enzyme binding site. When these single mutations were combined, the resulting multiple enzyme mutants are better than either parent. These single or multiple mutations also alter the relative pH activity profiles of the enzymes as shown in Figure 23.

To isolate the contribution of electrostatics to substrate specificity from other chemical binding forces, these various single and double mutants were analyzed for their ability to bind and cleave synthetic substrates containing Glu, Gln, Met and Lys as the P-1 substrate amino acid. This permitted comparisons between side-chains that were more sterically similar but differed in charge (e.g., Glu versus Gln, Lys versus Met). Similarly, mutant enzymes were assayed against homologous P-1 substrates that were most sterically similar but differed in charge (Table XIV).

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TABLE XIV

Kinetics of Position 156/166 Subtilisins
Determined for Different P1 Substrates

Enzyme Position 156 166	Net Charge (b)	P-1 Substrate log kcat/Km (log 1/Km) (c)			
		Glu	Gln	Met	Lys
Glu Asp	-2	n.d.	3.02 (2.56)	3.93 (2.74)	4.23 (3.00)
Glu Glu	-2	n.d.	3.06 (2.91)	3.86 (3.28)	4.48 (3.69)
Glu Asn	-1	1.62 (2.22)	3.85 (3.14)	4.99 (3.85)	4.15 (2.88)
Glu Gln	-1	1.20 (2.12)	4.36 (3.64)	5.43 (4.36)	4.10 (3.15)
Gln Asp	-1	1.30 (1.79)	3.40 (3.08)	4.94 (3.87)	4.41 (3.22)
Ser Asp	-1	1.23 (2.13)	3.41 (3.09)	4.67 (3.68)	4.24 (3.07)
Glu Met	-1	1.20 (2.30)	3.89 (3.19)	5.64 (4.83)	4.70 (3.89)
Glu Ala	-1	n.d.	4.34 (3.55)	5.65 (4.46)	4.90 (3.24)
Glu Gly (wt)	-1	1.20 (1.47)	3.85 (3.35)	5.07 (3.97)	4.60 (3.13)
Gln Gly	0	2.42 (2.48)	4.53 (3.81)	5.77 (4.61)	3.76 (2.82)
Ser Gly	0	2.31 (2.73)	4.09 (3.68)	5.61 (4.55)	3.46 (2.74)
Gln Asn	0	2.04 (2.72)	4.51 (3.76)	5.79 (4.66)	3.75 (2.74)
Ser Asn	0	1.91 (2.78)	4.57 (3.82)	5.72 (4.64)	3.68 (2.80)
Glu Arg	0	2.91 (3.30)	4.26 (3.50)	5.32 (4.22)	3.19 (2.80)
Glu Lys	0	4.09 (4.25)	4.70 (3.88)	6.15 (4.45)	4.23 (2.93)
Gln Lys	+1	4.70 (4.50)	4.64 (3.68)	5.97 (4.68)	3.23 (2.75)
Ser Lys	+1	4.21 (4.40)	4.84 (3.94)	6.16 (4.90)	3.73 (2.84)

Maximum difference:

log kcat/Km (log 1/Km) (d)

3.5 (3.0)	1.8 (1.4)	2.3 (2.2)	-1.3 (-1.0)
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Footnotes to Table XIV:

- (a) *B. subtilis*, BG 2036, expressing indicated variant subtilisin were fermented and enzymes purified as previously described (Estell, *et al.* (1985) *J. Biol. Chem.* 260, 6518-6521). Wild type subtilisin is indicated (wt) containing Glu156 and Gly166.
- (b) Net charge in the P-1 binding site is defined as the sum of charges from positions 156 and 166 at pH 8.6.
- (c) Values for $k_{cat}(s^{-1})$ and $K_m(M)$ were measured in 0.1M Tris pH 8.6 at 25°C as previously described against P-1 substrates having the form succinyl-L-AlaL-AlaL-ProL-[X]-p-nitroanilide, where X is the indicated P-1 amino acid. Values for log 1/ K_m are shown inside parentheses. All errors in determination of k_{cat}/K_m and 1/ K_m are below 5%.
- (d) Because values for Glu156/Asp166(D166) are too small to determine accurately, the maximum difference taken for GluP-1 substrate is limited to a charge range of +1 to -1 charge change.
- n.d. = not determined

The k_{cat}/K_m ratios shown are the second order rate constants for the conversion of substrate to product, and represent the catalytic efficiency of the enzyme. These ratios are presented in logarithmic form to scale the data, and because log k_{cat}/K_m is proportional to the lowering of transition-state activation energy (ΔG_T). Mutations at position 156 and 166 produce changes in catalytic efficiency toward Glu, Gln, Met and Lys P-1 substrates of 3100, 60, 200 and 20 fold, respectively. Making the P-1 binding-site more positively charged [e.g., compare Gln156/Lys166 (Q156/K166) versus Glu156/Met166 (Glu156/M166)] dramatically increased k_{cat}/K_m toward the Glu P-1 substrate (up to 3100 fold), and decreased the catalytic efficiency toward the Lys P-1 substrate (up to 10 fold). In addition, the results show that the catalytic efficiency of wild type enzyme can be

greatly improved toward any of the four P-1 substrates by mutagenesis of the P-1 binding site.

5 The changes in k_{cat}/K_m are caused predominantly by changes in $1/K_m$. Because $1/K_m$ is approximately equal to $1/K_s$, the enzyme-substrate association constant, the mutations primarily cause a change in substrate
10 binding. These mutations produce smaller effects on k_{cat} that run parallel to the effects on $1/K_m$. The changes in k_{cat} suggest either an alteration in binding in the P-1 binding site in going from the Michaelis-complex E·S to the transition-state complex (E·S[‡]) as previously proposed (Robertus, J.D., et al. (1972) Biochemistry 11, 2439-2449; Robertus, J.D., et al. (1972) Biochemistry 11, 4293-4303), or change in
15 the position of the scissile peptide bond over the catalytic serine in the E·S complex.

Changes in substrate preference that arise from changes in the net charge in the P-1 binding site show trends that are best accounted for by electrostatic
20 effects (Figure 28). As the P-1 binding cleft becomes more positively charged, the average catalytic efficiency increases much more for the Glu P-1 substrate than for its neutral and isosteric P-1 homolog, Gln (Figure 28A). Furthermore, at the
25 positive extreme both substrates have nearly identical catalytic efficiencies.

In contrast, as the P-1 site becomes more positively charged the catalytic efficiency toward the Lys P-1
30 substrate decreases, and diverges sharply from its neutral and isosteric homolog, Met (Figure 28B). The similar and parallel upward trend seen with increasing positive charge for the Met and Glu P-1 substrates probably results from the fact that all the substrates
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are succinylated on their amino-terminal end, and thus carry a formal negative charge.

5 The trends observed in $\log k_{cat}/K_m$ are dominated by changes in the K_m term (Figures 28C and 28D). As the pocket becomes more positively charged, the $\log 1/K_m$ values converge for Glu and Gln P-1 substrates (Figure 28C), and diverge for Lys and Met P-1 substrates (Figure 28D). Although less pronounced effects are seen in $\log k_{cat}$, the effects of P-1 charge on $\log k_{cat}$ parallel those seen in $\log 1/K_m$ and become larger as the P-1 pocket becomes more positively charged. This may result from the fact that the transition-state is a tetrahedral anion, and a net positive charge in the enzyme may serve to provide some added stabilization to the transition-state.

15 The effect of the change in P-1 binding-site charge on substrate preference can be estimated from the differences in slopes between the charged and neutral isosteric P-1 substrates (Figure 28B). The average change in substrate preference ($\Delta \log k_{cat}/K_m$) between charged and neutral isosteric substrates increases roughly 10-fold as the complementary charge or the enzyme increases (Table XV). When comparing Glu versus Lys, this difference is 100-fold and the change in substrate preference appears predominantly in the K_m term.

TABLE XV

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Differential Effect on Binding Site
Charge on log kcat/Km or (log 1/Km)
for P-1 Substrates that Differ in Charge (a)

5	Change in P-1 Binding Site Charge (b)	$\Delta \log \text{ kcat/Km}$		$(\Delta \log 1/\text{Km})$ GluLys
		GluGln	MetLys	
	-2 to -1	n.d.	1.2 (1.2)	n.d.
	-1 to 0	0.7 (0.6)	1.3 (0.8)	2.1 (1.4)
	0 to +1	1.5 (1.3)	0.5 (0.3)	2.0 (1.5)
10	Avg. change in log kcat/K or (log 1/Km) ^m per unit charge change	1.1 (1.0)	1.0 (0.8)	2.1 (1.5)

15 (a) The difference in the slopes of curves were taken between the P-1 substrates over the charge interval given for log (kcat/Km) (Figure 28A, B) and (log 1/Km) (Figure 28C, D). Values represent the differential effect a charge change has in distinguishing the substrates that are compared.

20 (b) Charge in P-1 binding site is defined as the sum of charges from positions 156 and 166.

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The free energy of electrostatic interactions in the structure and energetics of salt-bridge formation depends on the distance between the charges and the microscopic dielectric of the media. To dissect these structural and microenvironmental effects, the energies involved in specific salt-bridges were evaluated. In addition to the possible salt-bridges shown (Figures 29A and 29B), reasonable salt-bridges can be built between a Lys P-1 substrate and Asp at position 166, and between a Glu P-1 substrate and a Lys at position 166 (not shown). Although only one of these structures is confirmed by X-ray crystallography (Poulos, T.L., et al. (1976) J. Mol. Biol. 257 1097-1103), all models have favorable torsion angles (Sielecki, A.R., et al. (1979) J. Mol. Biol. 134, 781-804), and do not introduce unfavorable van der Waals contacts.

The change in charged P-1 substrate preference brought about by formation of the model salt-bridges above are shown in Table XVI.

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TABLE XVI

Effect of Salt Bridge Formation Between Enzyme
and Substrate on P1 Substrate Preference (a)

Enzymes Compared (b)		Enzyme Position Changed	P-1 Substrates Compared	Substrate (d) Preference $\Delta \log$ (kcat/Km)		Change in Substrate Preference $\Delta \Delta \log$ (kcat/Km)
1	2			1	2	
Glu156/Asp166	Gln156/Asp166	156	LysMet	+0.30	-0.53	0.83
Glu156/Asn166	Gln156/Asn166	156	LysMet	-0.84	-2.04	1.20
Glu156/Gly166	Gln156/Gly166	156	LysMet	-0.47	-2.10	1.63
Glu156/Lys-166	Gln156/Lys166	156	LysMet	-1.92	-2.74	0.82

-96-

Ave $\Delta \Delta \log$ (kcat/Km) 1.10 ± 0.3

Glu156/Asp166	Glu156/Asn166	166	LysMet	+0.30	-0.84	1.14
Glu156/Glu166	Glu156/Glu166	166	LysMet	+0.62	-1.33	1.95
Gln156/Asp166	Gln156/Asn166	166	LysMet	-0.53	-2.04	1.51
Ser156/Asp166	Ser156/Asn166	166	LysMet	-0.43	-2.04	1.61
Glu156/Lys166	Glu156/Met166	166	GluGln	-0.63	-2.69	2/06

Ave $\Delta \Delta \log$ (kcat/Km) 1.70 ± 0.3

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Footnotes to Table XVI:

(a) Molecular modeling shows it is possible to form a salt bridge between the indicated charged P-1 substrate and a complementary charge in the P-1 binding site of the enzyme at the indicated position changed.

5 (b) Enzymes compared have sterically similar amino acid substitutions that differ in charge at the indicated position.

(c) The P-1 substrates compared are structurally similar but differ in charge. The charged P-1 substrate is complementary to the charge change at the position indicated between enzymes 1 and 2.

10 (d) Data from Table XIV was used to compute the difference in $\log(k_{cat}/K_m)$ between the charged and the non-charged P-1 substrate (i.e., the substrate preference). The substrate preference is shown separately for enzyme 1 and 2.

15 (e) The difference in substrate preference between enzyme 1 (more highly charged) and enzyme 2 (more neutral) represents the rate change accompanying the electrostatic interaction.

The difference between catalytic efficiencies (i.e., $\Delta \log k_{cat}/K_m$) for the charged and neutral P-1 substrates (e.g., Lys minus Met or Glu minus Gln) give the substrate preference for each enzyme. The change in substrate preference ($\Delta \Delta \log k_{cat}/K_m$) between the charged and more neutral enzyme homologs (e.g., Glu156/Gly166 minus Gln156(Q156)/Gly166) reflects the change in catalytic efficiency that may be attributed solely to electrostatic effects.

30 These results show that the average change in substrate preference is considerably greater when electrostatic substitutions are produced at position 166 (50-fold in k_{cat}/K_m) versus position 156 (12-fold in k_{cat}/K_m). From these $\Delta \log k_{cat}/K_m$ values, an average change in transition-state stabilization energy can be calculated of -1.5 and -2.4 kcal/mol for

substitutions at positions 156 and 166, respectively. This should represent the stabilization energy contributed from a favorable electrostatic interaction for the binding of free enzyme and substrate to form the transition-state complex.

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EXAMPLE 10

Substitutions at Position 217

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Tyr217 has been substituted by all other 19 amino acids. Cassette mutagenesis as described in EPO publication No. 0130756 was used according to the protocol of Figure 22. The EcoRV restriction site was used for restriction-purification of pA217.

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Since this position is involved in substrate binding, mutations here effect kinetic parameters of the enzyme. An example is the substitution of Leu for Tyr at position 217. For the substrate SAAPFpNa, this mutant has a k_{cat} of 277 s^{-1} and a K_m of 4.7×10^{-4} with a k_{cat}/K_m ratio of 6×10^5 . This represents a 5.5-fold increase in k_{cat} with a 3-fold increase in K_m over the wild type enzyme.

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In addition, replacement of Tyr217 by Lys, Arg, Phe or Leu results in mutant enzymes which are more stable at pHs of about 9-11 than the WT enzyme. Conversely, replacement of Tyr217 by Asp, Glu, Gly or Pro results in enzymes which are less stable at pHs of about 9-11 than the WT enzyme.

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EXAMPLE 11

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Multiple Mutants Having
Altered Thermal Stability

5 B. amyloliquefacien subtilisin does not contain any
cysteine residues. Thus, any attempt to produce
thermal stability by Cys cross-linkage required the
substitution of more than one amino acid in subtilisin
with Cys. The following subtilisin residues were
multiply substituted with cysteine:

10 Thr22/Ser87
Ser24/Ser87

15 Mutagenesis of Ser24 to Cys was carried out with a 5'
phosphorylated oligonucleotide primer having the
sequence

5'-PC-TAC-ACT-GGA-TGC^{**}-AAT-GTT-AAA-G-3'.

20 (Asterisks show the location of mismatches and the
underlined sequence shows the position of the altered
Sau3A site.) The B. amyloliquefaciens subtilisin gene
on a 1.5 kb EcoRI-BAMHI fragment from pS4.5 was cloned
into M13mp11 and single stranded DNA was isolated.
This template (M13mp11SUBT) was double primed with the
25 5' phosphorylated M13 universal sequencing primer and
the mutagenesis primer. Adelman, et al. (1983) DNA 2,
183-193. The heteroduplex was transfected into
competent JM101 cells and plaques were probed for the
mutant sequence (Zoller, M.J., et al. (1982) Nucleic
30 Acid Res. 10, 6487-6500; Wallace, et al. (1981)
Nucleic Acid Res. 9, 3647-3656) using a
tetramethylammonium chloride hybridization protocol
(Wood, et al. (1985) Proc. Natl. Acad. Sci. USA 82,
1585-1588). The Ser87 to Cys mutation was prepared in
35

a similar fashion using a 5' phosphorylated primer having the sequence

5'-pGGC-GTT-GCG-CCA-TGC-GCA-TCA-CT-3'.

5 (The asterisk indicates the position of the mismatch and the underlined sequence shows the position of a new MstI site.) The C24 and C87 mutations were obtained at a frequency of one and two percent, respectively. Mutant sequences were confirmed by dideoxy sequencing in M13.

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Mutagenesis of Tyr21/Thr22 to A21/C22 was carried out with a 5' phosphorylated oligonucleotide primer having the sequence

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5'-pAC-TCT-CAA-GGC-^{***}GCT-^{**}TGT-GGC-TCA-AAT-GTT-3'.

20 (The asterisks show mismatches to the wild type sequence and the underlined sequence shows the position of an altered Sau3A site.) Manipulations for heteroduplex synthesis were identical to those described for C24. Because direct cloning of the heteroduplex DNA fragment can yield increased frequencies of mutagenesis, the EcoRI-BamHI subtilisin fragment was purified and ligated into pBS42. E. coli
25 MM 294 cells were transformed with the ligation mixture and plasmid DNA was purified from isolated transformants. Plasmid DNA was screened for the loss of the Sau3A site at codon 23 that was eliminated by the mutagenesis primer. Two out of 16 plasmid
30 preparations had lost the wild type Sau3A site. The mutant sequence was confirmed by dideoxy sequencing in M13.

Double mutants, C22/C87 and C24/C87, were constructed by ligating fragments sharing a common ClaI site that separated the single parent cystine codons. Specifically, the 500 bp EcoRI-ClaI fragment containing the 5' portion of the subtilisin gene (including codons 22 and 24) was ligated with the 4.7 kb ClaI-EcoRI fragment that contained the 3' portion of the subtilisin gene (including codon 87) plus pBS42 vector sequence. E. coli MM 294 was transformed with ligation mixtures and plasmid DNA was purified from individual transformants. Double-cysteine plasmid constructions were identified by restriction site markers originating from the parent cysteine mutants (i.e., C22 and C24, Sau3A minus; Cys87, MstI plus). Plasmids from E. coli were transformed into B. subtilis BG2036. The thermal stability of these mutants as compared to wild type subtilisin are presented in Figure 30 and Tables XVII and XVIII.

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TABLE XVII

Effect of DTT on the Half-Time of
Autolytic Inactivation of Wild-Type
and Disulfide Mutants of Subtilisin*

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Enzyme	$t_{1/2}$		-DTT/+DTT
	-DTT	+DTT	
	min		
Wild-type	95	85	1.1
C22/C87	44	25	1.8
C24/C87	92	62	1.5

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(*) Purified enzymes were either treated or not treated with 25mM DTT and dialyzed with or without 10mM DTT in 2mM CaCl_2 , 50mM Tris (pH 7.5) for 14 hr. at 4°C. Enzyme concentrations were adjusted to 80 μ l aliquots were quenched on ice and assayed for residual activity. Half-times for autolytic inactivation were determined from semi-log plots of \log_{10} (residual activity) versus time. These plots were linear for over 90% of the inactivation.

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TABLE XVIII

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Effect of Mutations in Subtilisin
on the Half-Time of Autolytic
Inactivation at 58°C*

	Enzyme	$t_{1/2}$ min
5	Wild-type	
	C22	120
	C24	22
	C87	120
10	C22/C87	104
	C24/C87	43
		115

(*) Half-times for autolytic inactivation were determined for wild-type and mutant subtilisins as described in the legend to Table III. Unpurified and non-reduced enzymes were used directly from B. subtilis culture supernatants.

The disulfides introduced into subtilisin did not improve the autolytic stability of the mutant enzymes when compared to the wild-type enzyme. However, the disulfide bonds did provide a margin of autolytic stability when compared to their corresponding reduced double-cysteine enzyme. Inspection of a highly refined x-ray structure of wild-type B. amyloliquefaciens subtilisin reveals a hydrogen bond between Thr22 and Ser87. Because cysteine is a poor hydrogen donor or acceptor (Paul, I.C. (1974) in Chemistry of the -SH Group (Patai, S., ed.) pp. 111-149, Wiley Interscience, New York) weakening of 22/87 hydrogen bond may explain why the C22 and C87 single-cysteine mutant proteins are less autolytically stable than either C24 or wild-type (Table XVIII). The fact that C22 is less autolytically stable than C87 may be the result of the Tyr21A mutation (Table XVIII). Indeed,

construction and analysis of Tyr21/C22 shows the mutant protein has an autolytic stability closer to that of C87. In summary, the C22 and C87 of single-cysteine mutations destabilize the protein toward autolysis, and disulfide bond formation increases the stability to a level less than or equal to that of wild-type enzyme.

EXAMPLE 12

Multiple Mutants Containing Substitutions at Position 222 and Position 166 or 169

Double mutants 166/222 and 169/222 were prepared by ligating together (1) the 2.3kb AcaII fragment from pS4.5 which contains the 5' portion of the subtilisin gene and vector sequences, (2) the 200bp AvaII fragment which contains the relevant 166 or 169 mutations from the respective 166 or 169 plasmids, and (3) the 2.2kb AvaII fragment which contains the relevant 222 mutation 3' and of the subtilisin genes and vector sequence from the respective p222 plasmid.

Although mutations at position 222 improve oxidation stability they also tend to increase the K_m . An example is shown in Table XIX. In this case the A222 mutation was combined with the K166 mutation to give an enzyme with k_{cat} and K_m intermediate between the two parent enzymes.

TABLE XIX

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	<u>kcat</u>	<u>Km</u>
WT	50	1.4×10^{-4}
A222	42	9.9×10^{-4}
K166	21	3.7×10^{-5}
K166/A222	29	2.0×10^{-4}

substrate sAAPFPNa

EXAMPLE 13

Multiple Mutants Containing
Substitutions at Positions 50, 156,
166, 217 and Combinations Thereof

The double mutant S156/A169 was prepared by ligation of two fragments, each containing one of the relevant mutations. The plasmid pS156 was cut with XmaI and treated with S1 nuclease to create a blunt end at codon 167. After removal of the nuclease by phenol/chloroform extraction and ethanol precipitation, the DNA was digested with BamHI and the approximately 4kb fragment containing the vector plus the 5' portion of the subtilisin gene through codon 167 was purified.

The pA169 plasmid was digested with KpnI and treated with DNA polymerase Klenow fragment plus 50 μ M dNTPs to create a blunt end codon at codon 168. The Klenow was removed by phenol/chloroform extraction and ethanol precipitation. The DNA was digested with BamHI and the 590bp fragment including codon 168 through the carboxy terminus of the subtilisin gene

was isolated. The two fragments were then ligated to give S156/A169.

Triple and quadruple mutants were prepared by ligating together (1) the 220bp PvuII/HaeII fragment containing the relevant 156, 166 and/or 169 mutations from the respective p156, p166 and/or p169 double of single mutant plasmid, (2) the 550bp HaeII/BamHI fragment containing the relevant 217 mutant from the respective p217 plasmid, and (3) the 3.9kb PvuII/BamHI fragment containing the F50 mutation and vector sequences.

The multiple mutant F50/S156/A169/L217, as well as B. amyloliquefaciens subtilisin, B. licheniformis subtilisin and the single mutant L217 were analyzed with the above synthetic polypeptides where the P-1 amino acid in the substrate was Lys, His, Ala, Gln, Tyr, Phe, Met and Leu. These results are shown in Figures 26 and 27.

These results show that the F50/S156/A169/L217 mutant has substrate specificity similar to that of the B. licheniformis enzyme and differs dramatically from the wild type enzyme. Although only data for the L217 mutant are shown, none of the single mutants (e.g., F50, S156 or A169) showed this effect. Although B. licheniformis differs in 88 residue positions from B. amyloliquefaciens, the combination of only these four mutations accounts for most of the differences in substrate specificity between the two enzymes.

EXAMPLE 14

Subtilisin Mutants Having Altered Alkaline Stability

A random mutagenesis technique was used to generate single and multiple mutations within the B.

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amyloliquefaciens subtilisin gene. Such mutants were screened for altered alkaline stability. Clones having increased (positive) alkaline stability and decreased (negative) alkaline stability were isolated and sequenced to identify the mutations within the subtilisin gene. Among the positive clones, the mutants V107 and R213 were identified. These single mutants were subsequently combined to produce the mutant V107/R213.

One of the negative clones (V50) from the random mutagenesis experiments resulted in a marked decrease in alkaline stability. Another mutant (P50) was analyzed for alkaline stability to determine the effect of a different substitution at position 50. The F50 mutant was found to have a greater alkaline stability than wild type subtilisin and when combined with the double mutant V107/R213 resulted in a mutant having an alkaline stability which reflected the aggregate of the alkaline stabilities for each of the individual mutants.

The single mutant R204 and double mutant C204/R213 were identified by alkaline screening after random cassette mutagenesis over the region from position 197 to 228. The C204/R213 mutant was thereafter modified to produce mutants containing the individual mutations C204 and R213 to determine the contribution of each of the individual mutations. Cassette mutagenesis using pooled oligonucleotides to substitute all amino acids at position 204, was utilized to determine which substitution at position 204 would maximize the increase in alkaline stability. The mutation from Lys213 to Arg was maintained constant for each of these substitutions at position 204.

A. Construction of pB0180, an
E. coli-B. subtilis Shuttle Plasmid

The 2.9 kb EcoRI-BamHI fragment from pBR327 (Covarrubias, L., et al. (1981) Gene 13, 25-35) was ligated to the 3.7kb EcoRI-BamHI fragment of pBD64 (Gryczan, T., et al. (1980) J. Bacteriol., 141, 246-253) to give the recombinant plasmid pB0153. The unique EcoRI recognition sequence in pBD64 was eliminated by digestion with EcoRI followed by treatment with Klenow and deoxynucleotide triphosphates (Maniatis, T., et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). Blunt end ligation and transformation yielded pB0154. The unique AvaI recognition sequence in pB0154 was eliminated in a similar manner to yield pB0171. pB0171 was digested with BamHI and PvuII and treated with Klenow and deoxynucleotide triphosphates to create blunt ends. The 6.4 kb fragment was purified, ligated and transformed into LE392 cells (Enquest, L.W., et al. (1977) J. Mol. Biol. 111, 97-120), to yield pB0172 which retains the unique BamHI site. To facilitate subcloning of subtilisin mutants, a unique and silent KpnI site starting at codon 166 was introduced into the subtilisin gene from pS4.5 (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925) by site-directed mutagenesis. The KpnI+ plasmid was digested with EcoRI and treated with Klenow and deoxynucleotide triphosphates to create a blunt end. The Klenow was inactivated by heating for 20 min at 68°C, and the DNA was digested with BamHI. The 1.5 kb blunt EcoRI-BamHI fragment containing the entire subtilisin was ligated with the 5.8 kb NruI-BamHI from pB0172 to yield pB0180. The ligation of the blunt NruI end to the blunt EcoRI end recreated an EcoRI

site. Proceeding clockwise around pB0180 from the EcoRI site at the 5' end of the subtilisin gene is the unique BamHI site at the 3' end of the subtilisin gene, the chloramphenicol and neomycin resistance genes and UB110 gram positive replication origin derived from pBD64, the ampicillin resistance gene and gram negative replication origin derived from pBR327.

B. Construction of Random Mutagenesis Library

The 1.5 kb EcoRI-BamHI fragment containing the *B. amyloliquefaciens* subtilisin gene (Wells et al., 1983) from pB0180 was cloned into M13mp11 to give M13mp11 SUBT essentially as previously described (Wells, J.A., et al. (1986) J. Biol. Chem., 261,6564-6570). Deoxyuridine containing template DNA was prepared according to Kunkel (Kunkel, T.A. (1985) Proc. Natl. Acad. Sci. USA, 82 488-492). Uridine containing template DNA (Kunkel, 1985) was purified by CsCl density gradients (Maniatis, T. et al. (eds.) (1982) in Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.). A primer (AvaI⁻) having the sequence

5'GAAAAAAGACCCTAGCGTCGCTTA

ending at codon -11, was used to alter the unique AvaI recognition sequence within the subtilisin gene. (The asterisk denotes the mismatches from the wild-type sequence and underlined is the altered AvaI site.)

The 5' phosphorylated AvaI primer (~320 pmol) and ~40 pmol (~120µg) of uridine containing M13mp11 SUBT template in 1.88 ml of 53 mM NaCl, 7.4 mM MgCl₂ and 7.4 mM Tris.HCl (pH 7.5) were annealed by heating to

90°C for 2 min. and cooling 15 min at 24°C (Fig. 31). Primer extension at 24°C was initiated by addition of 100µL containing 1 mM in all four deoxynucleotide triphosphates, and 20µl Klenow fragment (5 units/l). The extension reaction was stopped every 15 seconds over ten min by addition of 10µl 0.25 M EDTA (pH 8) to 50µl aliquots of the reaction mixture. Samples were pooled, phenol chlorophorm extracted and DNA was precipitated twice by addition of 2.5 vol 100% ethanol, and washed twice with 70% ethanol. The pellet was dried, and redissolved in 0.4 ml 1 mM EDTA, 10 mM Tris (pH 8).

Misincorporation of α-thiodeoxynucleotides onto the 3' ends of the pool of randomly terminated template was carried out by incubating four 0.2 ml solutions each containing one-fourth of the randomly terminated template mixture (~20µg), 0.25 mM of a given α-thiodeoxynucleotide triphosphate, 100 units AMV polymerase, 50 mM KCL, 10 mM MgCl₂, 0.4 mM dithiothreitol, and 50 mM Tris (pH 8.3) (Champoux, J.J. (1984) Genetics, 2, 454-464). After incubation at 37°C for 90 minutes, misincorporation reactions were sealed by incubation for five minutes at 37°C with 50 mM all four deoxynucleotide triphosphates (pH 8), and 50 units AMV polymerase. Reactions were stopped by addition of 25 mM EDTA (final), and heated at 68°C for ten min to inactivate AMV polymerase. After ethanol precipitation and resuspension, synthesis of closed circular heteroduplexes was carried out for two days at 14°C under the same conditions used for the timed extension reactions above, except the reactions also contained 1000 units T4 DNA ligase, 0.5 mM ATP and 1 mM β-mercaptoethanol. Simultaneous restriction of each heteroduplex pool with KpnI, BamHI, and EcoRI confirmed that the

extension reactions were nearly quantitative. Heteroduplex DNA in each reaction mixture was methylated by incubation with 80 μ M S-adenosylmethionine and 150 units dam methylase for 1 hour at 37°C. Methylation reactions were stopped by heating at 68°C for 15 min.

One-half of each of the four methylated heteroduplex reactions were transformed into 2.5 ml competent E. coli JM101 (Messing, J. (1979) Recombinant DNA Tech. Bull., 2, 43-48). The number of independent transformants from each of the four transformations ranged from 0.4-2.0 $\times 10^5$. After growing out phage pools, RF DNA from each of the four transformations was isolated and purified by centrifugation through CsCl density gradients. Approximately 2 μ g of RF DNA from each of the four pools was digested with EcoRI, BamHI and AvaI. The 1.5 kb EcoRI-BamHI fragment (i.e., AvaI resistant) was purified on low gel temperature agarose and ligated into the 5.5 kb EcoRI-BamHI vector fragment of pB0180. The total number of independent transformants from each α -thiodeoxynucleotide misincorporation plasmid library ranged from 1.2-2.4 $\times 10^4$. The pool of plasmids from each of the four transformations was grown out in 200 ml LB media containing 12.5 μ g/ml cmp and plasmid DNA was purified by centrifugation through CsCl density gradients.

C. Expression and Screening of Subtilisin Point Mutants

Plasmid DNA from each of the four misincorporation pools was transformed (Anagnostopoulos, C., et al. (1967), J. Bacteriol., 81, 741-746) into BG2036. For each transformation, 5 μ g of DNA produced approximately

2.5 x 10⁵ independent BG2036 transformants, and liquid culture aliquots from the four libraries were stored in 10% glycerol at 70°C. Thawed aliquots of frozen cultures were plated on LB/5µg/ml cmp/1.6% skim milk plates (Wells, J.A., et al. (1983) Nucleic Acids Res., 11, 7911-7925), and fresh colonies were arrayed onto 96-well microtiter plates containing 150 µl per well LB media plus 12.5µg/ml cmp. After 1 h at room temperature, a replica was stamped (using a matched 96 prong stamp) onto a 132 mm BA 85 nitrocellulose filter (Schleicher and Scheull) which was layered on a 140 mm diameter LB/cmp/skim milk plate. Cells were grown about 16 h at 30°C until halos of proteolysis were roughly 5-7 mm in diameter and filters were transferred directly to a freshly prepared agar plate at 37°C containing only 1.6% skim milk and 50 mM sodium phosphate pH 11.5. Filters were incubated on plates for 3-6 h at 37°C to produce halos of about 5 mm for wild-type subtilisin and were discarded. The plates were stained for 10 min at 24°C with Coomassie blue solution (0.25% Coomassie blue (R-250) 25% ethanol) and destained with 25% ethanol, 10% acetic acid for 20 min. Zones of proteolysis appeared as blue halos on a white background on the underside of the plate and were compared to the original growth plate that was similarly stained and destained as a control. Clones were considered positive that produced proportionately larger zones of proteolysis on the high pH plates relative to the original growth plate. Negative clones gave smaller halos under alkaline conditions. Positive and negative clones were restreaked to colony purify and screened again in triplicate to confirm alkaline pH results.

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D. Identification and Analysis
of Mutant Subtilisins

Plasmid DNA from 5 ml overnight cultures of more
alkaline active *B. subtilis* clones was prepared
according to Birnboim and Doly (Birnboim, H.C., et al.
(1979) Nucleic Acid Res. 7, 1513) except that
5 incubation with 2 mg/ml lysozyme proceeded for 5 min
at 37°C to ensure cell lysis and an additional
phenol/CHCl₃ extraction was employed to remove
contaminants. The 1.5 kb EcoRI-BamHI fragment
containing the subtilisin gene was ligated into
10 M13mp11 and template DNA was prepared for DNA
sequencing (Messing, J., et al. (1982) Gene, 19
269-276). Three DNA sequencing primers ending at codon
26, +95, and +155 were synthesized to match the
subtilisin coding sequence. For preliminary sequence
15 identification a single track of DNA sequence,
corresponding to the dNTPas misincorporation library
from which the mutant came, was applied over the
entire mature protein coding sequence (i.e., a single
dideoxyguanosine sequence track was applied to
20 identify a mutant from the dGTPas library). A
complete four track of DNA sequence was performed 200
bp over the site of mutagenesis to confirm and
identify the mutant sequence (Sanger, F., et al.,
(1980) J. Mol. Biol., 143, 161-178). Confirmed
25 positive and negative bacilli clones were cultured in
LB media containing 12.5 µg/mL cmp and purified from
culture supernatants as previously described (Estell,
D.A., et al. (1985) J. Biol. Chem., 260, 6518-6521).
Enzymes were greater than 98% pure as analyzed by
30 SDS-polyacrylamide gel electrophoresis (Laemmli, U.K.
(1970), Nature, 227, 680-685), and protein
concentrations were calculated from the absorbance at
280 nm, $\epsilon_{280}^{0.1\%} = 1.17$ (Maturbara, H., et al. (1965), J.
Biol. Chem., 240, 1125-1130).

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Enzyme activity was measured with 200 μ g/mL succinyl-L-AlaL-AlaL-ProL-Phep-nitroanilide (Sigma) in 0.1M Tris pH 8.6 or 0.1 M CAPS pH 10.8 at 25°C. Specific activity (μ moles product/min-mg) was calculated from the change in absorbance at 410 nm from production of p-nitroaniline with time per mg of enzyme (E410 = 8,480 M-lcm-1; Del Mar, E.G., et al. (1979), Anal. Biochem., 99, 316-320). Alkaline autolytic stability studies were performed on purified enzymes (200 μ g/mL) in 0.1 M potassium phosphate (pH 12.0) at 37°C. At various times aliquots were assayed for residual enzyme activity (Wells, J.A., et al. (1986) J. Biol. Chem., 261, 6564-6570).

15 E. Results

1. Optimization and analysis of mutagenesis frequency

A set of primer-template molecules that were randomly 3'-terminated over the subtilisin gene (Fig. 31) was produced by variable extension from a fixed 5'-primer (The primer mutated a unique *Ava*I site at codon 11 in the subtilisin gene). This was achieved by stopping polymerase reactions with EDTA after various times of extension. The extent and distribution of duplex formation over the 1 kb subtilisin gene fragment was assessed by multiple restriction digestion (not shown). For example, production of new *Hinf*I fragments identified when polymerase extension had proceeded past Ile110, Leu233, and Asp259 in the subtilisin gene.

Misincorporation of each dNTPs at randomly terminated 3' ends by AMV reverse transcriptase (Zakour, R.A., et al. (1982), Nature, 295, 708-710; Zakour, R.A., et al. (1984), Nucleic Acids Res., 12, 6615-6628). used

conditions previously described (Champoux, J.J., (1984), Genetics, 2, 454-464). The efficiency of each misincorporation reaction was estimated to be greater than 80% by the addition of each dNTPs to the AvaI restriction primer, and analysis by polyacrylamide gel electrophoresis. Misincorporations were sealed by polymerization with all four dNTP's and closed circular DNA was produced by reaction with DNA ligase.

Several manipulations were employed to maximize the yield of the mutant sequences in the heteroduplex. These included the use of a deoxyuridine containing template (Kunkel, T.A. (1985), Proc. Natl. Acad. Sci. USA, 82 488-492; Pukkila, P.J. et al. (1983), Genetics, 104, 571-582), in vitro methylation of the mutagenic strand (Kramer, W. et al. (1982) Nucleic Acids Res., 10 6475-6485), and the use of AvaI restriction-selection against the wild-type template strand which contained a unique AvaI site. The separate contribution of each of these enrichment procedures to the final mutagenesis frequency was not determined, except that prior to AvaI restriction-selection roughly one-third of the segregated clones in each of the four pools still retained a wild-type AvaI site within the subtilisin gene. After AvaI restriction-selection greater than 98% of the plasmids lacked the wild-type AvaI site.

The 1.5 kb EcoRI-BamHI subtilisin gene fragment that was resistant to AvaI restriction digestion, from each of the four CsCl purified M13 RF pools was isolated on low melting agarose. The fragment was ligated in situ from the agarose with a similarly cut E. coli-B. subtilis shuttle vector, pB0180, and transformed directly into E. coli LE392. Such direct ligation and transformation of DNA isolated from agarose avoided

loses and allowed large numbers of recombinants to be obtained (>100,000 per μ g equivalent of input M13 pool).

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The frequency of mutagenesis for each of the four dNTPs misincorporation reactions was estimated from the frequency that unique restriction sites were eliminated (Table XX). The unique restriction sites chosen for this analysis, ClaI, PvuII, and KpnI, were distributed over the subtilisin gene starting at codons 35, 104, and 166, respectively. As a control, the mutagenesis frequency was determined at the PstI site located in the β lactamase gene which was outside the window of mutagenesis. Because the absolute mutagenesis frequency was close to the percentage of undigested plasmid DNA, two rounds of restriction-selection were necessary to reduce the background of surviving uncut wild-type plasmid DNA below the mutant plasmid (Table XX). The background of surviving plasmid from wild-type DNA probably represents the sum total of spontaneous mutations, uncut wild-type plasmid, plus the efficiency with which linear DNA can transform E. coli. Subtracting the frequency for unmutagenized DNA (background) from the frequency for mutant DNA, and normalizing for the window of mutagenesis sampled by a given restriction analysis (4-6 bp) provides an estimate of the mutagenesis efficiency over the entire coding sequence (~1000 bp).

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TABLE XX

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	α -thiol dNTP misincor- porated (b)	Restriction Site Selection	% resistant clones ^c			% resistant clones over Background ^d	% mutants per 1000bp ^e
			1st round	2nd round	Total		
5	None	<u>PstI</u>	0.32	0.7	0.002	0	-
	G	<u>PstI</u>	0.33	1.0	0.003	0.001	0.2
	T	<u>PstI</u>	0.32	<0.5	<0.002	0	0
	C	<u>PstI</u>	0.43	3.0	0.013	0.011	3
10	None	<u>ClaI</u>	0.28	5	0.014	0	-
	G	<u>ClaI</u>	2.26	85	1.92	1.91	380
	T	<u>ClaI</u>	0.48	31	0.15	0.14	35
	C	<u>ClaI</u>	0.55	15	0.08	0.066	17
15	None	<u>PvuII</u>	0.08	29	0.023	0	-
	G	<u>PvuII</u>	0.41	90	0.37	0.35	88
	T	<u>PvuII</u>	0.10	67	0.067	0.044	9
	C	<u>PvuII</u>	0.76	53	0.40	0.38	95
20	None	<u>KpnI</u>	0.41	3	0.012	0	-
	G	<u>KpnI</u>	0.98	35	0.34	0.33	83
	T	<u>KpnI</u>	0.36	15	0.054	0.042	8
	C	<u>KpnI</u>	1.47	26	0.38	0.37	93

(a) Mutagenesis frequency is estimated from the frequency for obtaining mutations that alter unique restriction sites within the mutagenized subtilisin gene (i.e., ClaI, PvuII, or KpnI) compared to mutation frequencies of the PstI site, that is outside the window of mutagenesis.

(b) Plasmid DNA was from wild-type (none) or mutagenized by dNTPs misincorporation as described.

(c) Percentage of resistant clones was calculated from the fraction of clones obtained after three fold or greater over-digestion of the plasmid with the indicated restriction enzyme compared to a

non-digested control. Restriction-resistant plasmid DNA from the first round was subjected to a second round of restriction-selection. The total represents the product of the fractions of resistant clones obtained from both rounds of selection and gives percentage of restriction-site mutant clones in the original starting pool. Frequencies were derived from counting at least 20 colonies and usually greater than 100.

(d) Percent resistant clones was calculated by subtracting the percentage of restriction-resistant clones obtained for wild-type DNA (i.e., none) from that obtained for mutant DNA.

(e) This extrapolates from the frequency of mutation over each restriction site to the entire subtilisin gene (~1 kb). This has been normalized to the number of possible bases (4-6 bp) within each restriction site that can be mutagenized by a given misincorporation event.

From this analysis, the average percentage of subtilisin genes containing mutations that result from dGTPas, dCTPas, or dTTPas misincorporation was estimated to be 90, 70, and 20 percent, respectively. These high mutagenesis frequencies were generally quite variable depending upon the dNTPas and misincorporation efficiencies at this site. Misincorporation efficiency has been reported to be both dependent on the kind of mismatch, and the context of primer (Champoux, J.J., (1984); Skinner, J.A., et al. (1986) Nucleic Acids Res., 14, 6945-6964). Biased misincorporation efficiency of dGTPas and dCTPas over dTTPas has been previously observed (Shortle, D., et al. (1985), Genetics, 110, 539-555). Unlike the dGTPas, dCTPas, and dTTPas libraries the efficiency of mutagenesis for the dATPas

misincorporation library could not be accurately assessed because 90% of the restriction-resistant plasmids analyzed simply lacked the subtilisin gene insert. This problem probably arose from self-ligation of the vector when the dATPas mutagenized subtilisin gene was subcloned from M13 into pB0180. Correcting for the vector background, we estimate the mutagenesis frequency around 20 percent in the dATPas misincorporation library. In a separate experiment (not shown), the mutagenesis efficiencies for dGTPas and dTTPas misincorporation were estimated to be around 50 and 30 percent, respectively, based on the frequency of reversion of an inactivating mutation at codon 169.

The location and identity of each mutation was determined by a single track of DNA sequencing corresponding to the misincorporated α thiodeoxy-nucleotide over the entire gene followed by a complete four track of DNA sequencing focused over the site of mutation. Of 14 mutants identified, the distribution was similar to that reported by Shortle and Lin (1985) except we did not observe nucleotide insertion or deletion mutations. The proportion of AG mutations was highest in the G misincorporation library, and some unexpected point mutations appeared in the dTTPas and dCTPas libraries.

2. Screening and Identification of Alkaline Stability Mutants of Subtilisin

It is possible to screen colonies producing subtilisin by halos of casein digestion (Wells, J.A. et al. (1983) Nucleic Acids Res., 11, 7911-7925). However, two problems were posed by screening colonies under high alkaline conditions (> pH 11). First, B. subtilis

will not grow at high pH, and we have been unable to transform an alkylphilic strain of bacillus. This problem was overcome by adopting a replica plating strategy in which colonies were grown on filters at neutral pH to produce subtilisin and filters subsequently transferred to casein plates at pH 11.5 to assay subtilisin activity. However, at pH 11.5 the casein micells no longer formed a turbid background and thus prevented a clear observation of proteolysis halos. The problem was overcome by briefly staining the plate with Coomassie blue to amplify proteolysis zones and acidifying the plates to develop casein micell turbidity. By comparison of the halo size produced on the reference growth plate (pH 7) to the high pH plate (pH 11.5), it was possible to identify mutant subtilisins that had increased (positives) or decreased (negatives) stability under alkaline conditions.

Roughly 1000 colonies were screened from each of the four misincorporation libraries. The percentage of colonies showing a differential loss of activity at pH 11.5 versus pH 7 represented 1.4, 1.8, 1.4, and 0.6% of the total colonies screened from the thiol dGTPas, dATPas, dTTPas, and dCTPAs libraries, respectively. Several of these negative clones were sequenced and all were found to contain a single base change as expected from the misincorporation library from which they came. Negative mutants included A36, E170 and V50. Two positive mutants were identified as V107 and R213. The ratio of negatives to positives was roughly 50:1.

3. Stability and Activity of
Subtilisin Mutants at Alkaline pH

Subtilisin mutants were purified and their autolytic stabilities were measured by the time course of inactivation at pH 12.0 (Figs. 32 and 33). Positive mutants identified from the screen (i.e., V107 and R213) were more resistant to alkaline induced autolytic inactivation compared to wild-type; negative mutants (i.e., E170 and V50) were less resistant. We had advantageously produced another mutant at position 50 (F50) by site-directed mutagenesis. This mutant was more stable than wild-type enzyme to alkaline autolytic inactivation (Fig. 33) At the termination of the autolysis study, SDS-PAGE analysis confirmed that each subtilisin variant had autolyzed to an extent consistent with the remaining enzyme activity.

The stabilizing effects of V107, R213, and F50 are cumulative. See Table XXI. The double mutant, V107/R213 (made by subcloning the 920 bp EcoRI-KpnI fragment of pB0180V107 into the 6.6 kb EcoRI-KpnI fragment of pB0180R213), is more stable than either single mutant. The triple mutant, F50/V107/R213 (made by subcloning the 735 bp EcoRI-PvuII fragment of pF50 (Example 2) into the 6.8 kb EcoRI-PvuII fragment of pB0180/V107, is more stable than the double mutant V107/R213 or F50. The inactivation curves show a biphasic character that becomes more pronounced the more stable the mutant analyzed. This may result from some destabilizing chemical modification(s) (eg., deamidation) during the autolysis study and/or reduced stabilization caused by complete digestion of larger autolysis peptides. These alkaline autolysis studies have been repeated on separately purified enzyme batches with essentially the same results. Rates of autolysis should depend both on the conformational

F. Random Cassette Mutagenesis
of Residues 197 through 228

Plasmid pA222 (Wells, et al. (1985) Gene 34, 315-323) was digested with PstI and BamHI and the 0.4 kb PstI/BamHI fragment (fragment 1, see Fig. 34) purified from a polyacrylamide gel by electroelution.

The 1.5 kb EcoRI/BamHI fragment from pS4.5 was cloned into M13mp9. Site directed mutagenesis was performed to create the A197 mutant and simultaneously insert a silent SstI site over codons 195-196. The mutant EcoRI/BamHI fragment was cloned back into pBS42. The pA197 plasmid was digested with BamHI and SstI and the 5.3 kb BamHI/SstI fragment (fragment 2) was purified from low melting agarose.

Complimentary oligonucleotides were synthesized to span the region from SstI (codons 195-196) to PstI (codons 228-230). These oligodeoxynucleotides were designed to (1) restore codon 197 to the wild type, (2) re-create a silent KpnI site present in pA222 at codons 219-220, (3) create a silent SmaI site over codons 210-211, and (4) eliminate the PstI site over codons 228-230 (see Fig. 35). Oligodeoxynucleotides were synthesized with 2% contaminating nucleotides at each cycle of synthesis, e.g., dATP reagent was spiked with 2% dCTP, 2% dGTP, and 2% dTTP. For 97-mers, this 2% poisoning should give the following percentages of non-mutant, single mutants and double or higher mutants per strand with two or more misincorporations per complimentary strand: 14% non-mutant, 28% single mutant, and 57% with ≥2 mutations, according to the general formula

$$f = \frac{\mu^n}{n!} e^{-\mu}$$

where μ is the average number of mutations and n is a number class of mutations and f is the fraction of the total having that number of mutations. Complimentary oligodeoxynucleotide pools were phosphorylated and annealed (fragment 3) and then ligated at 2-fold molar excess over fragments 1 and 2 in a three-way ligation.

E. coli MM294 was transformed with the ligation reaction, the transformation pool grown up over night and the pooled plasmid DNA was isolated. This pool represented 3.4×10^4 independent transformants. This plasmid pool was digested with *Pst*I and then used to retransform *E. coli*. A second plasmid pool was prepared and used to transform *B. subtilis* (BG2036). Approximately 40% of the BG2036 transformants actively expressed subtilisin as judged by halo-clearing on casein plates. Several of the non-expressing transformants were sequenced and found to have insertions or deletions in the synthetic cassettes. Expressing BG2036 mutants were arrayed in microtiter dishes with 150 μ l of LB/12.5 μ g/mL chloramphenicol (cmp) per well, incubated at 37°C for 3-4 hours and then stamped in duplicate onto nitrocellulose filters laid on LB 1.5% skim milk/5 μ g/mL cmp plates and incubated overnight at 33°C (until halos were approximately 4-8 mm in diameter). Filters were then lifted to stacks of filter paper saturated with 1 x Tide commercial grade detergent, 50 mM Na₂CO₃, pH 11.5 and incubated at 65°C for 90 min. Overnight growth plates were Commassie stained and destained to establish basal levels of expression. After this treatment, filters were returned to pH7/skim milk/20 μ g/mL tetracycline plates and incubated at 37°C for 4 hours to overnight.

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5 Mutants identified by the high pH stability screen to be more alkaline stable were purified and analyzed for autolytic stability at high pH or high temperature. The double mutant C204/R213 was more stable than wild type at either high pH or high temperature (Table XXII).

10 This mutant was dissected into single mutant parents (C204 and R213) by cutting at the unique SmaI restriction site (Fig. 35) and either ligating wild type sequence 3' to the SmaI site to create the single C204 mutant or ligating wild type sequence 5' to the SmaI site to create the single R213 mutant. Of the two single parents, C204 was nearly as alkaline stable as the parent double mutant (C04/R213) and slightly more thermally stable. See Table XXII. The R213 mutant was only slightly more stable than wild type under both conditions (not shown).

20 Another mutant identified from the screen of the 197 to 228 random cassette mutagenesis was R204. This mutant was more stable than wild type at both high pH and high temperature but less stable than C204.

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30

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TABLE XXII

Stability of subtilisin variants

5 Purified enzymes (200 μ g/mL) were incubated in 0.1M phosphate, pH 12 at 30°C for alkaline autolysis, or in 2mM CaCl₂, 50mM MOPS, pH 7.0 at 62°C for thermal autolysis. At various times samples were assayed for residual enzyme activity. Inactivations were roughly pseudo-first order, and $t_{1/2}$ gives the time it took to reach 50% of the starting activity in two separate experiments.

Subtilisin variant	$t_{1/2}$ (alkaline autolysis)		$t_{1/2}$ (thermal autolysis)	
	Exp. #1	Exp. #2	Exp. #1	Exp. #2
wild type	30	25	20	23
F50/V107/R213	49	41	18	23
R204	35	32	24	27
C204	43	46	38	40
C204/R213	50	52	32	36
L204/R213	32	30	20	21

G. Random Mutagenesis at Codon 204

30 Based on the above results, codon 204 was targeted for random mutagenesis. Mutagenic DNA cassettes (for codon at 204) all contained a fixed R213 mutation which was found to slightly augment the stability of the C204 mutant.

Plasmid DNA encoding the subtilisin mutant C204/R213 was digested with SstI and EcoRI and a 1.0 kb EcoRI/SstI fragment was isolated by electro-elution from polyacrylamide gel (fragment 1, see Fig. 35).

C204/R213 was also digested with SmaI and EcoRI and the large 4.7 kb fragment, including vector sequences and the 3' portion of coding region, was isolated from low melting agarose (fragment 2, see Fig. 36).

Fragments 1 and 2 were combined in four separate three-way ligations with heterophosphorylated fragments 3 (see Figs. 36 and 37). This heterophosphorylation of synthetic duplexes should preferentially drive the phosphorylated strand into the plasmid ligation product. Four plasmid pools, corresponding to the four ligations, were restricted with SmaI in order to linearize any single cut C204/R213 present from fragment 2 isolation, thus reducing the background of C204/R213. E. coli was then re-transformed with SmaI-restricted plasmid pools to yield a second set of plasmid pools which are essentially free of C204/R213 and any non-segregated heterduplex material.

These second enriched plasmid pools were then used to transform B. subtilis (BG2036) and the resulting four mutant pools were screened for clones expressing subtilisin resistant to high pH/temperature inactivation. Mutants found positive by such a screen were further characterized and identified by sequencing.

The mutant L204/R213 was found to be slightly more stable than the wild type subtilisin. See Table XXII.

Having described the preferred embodiments of the present invention, it will appear to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments, and that such modifications are intended to be within the scope of the present invention.

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CLAIMS;

1. A carbonyl hydrolase mutant having at least one
property which is substantially different from the
same property of a precursor carbonyl hydrolase from
which the amino acid sequence of said carbonyl
5 hydrolase mutant is derived, said property being
selected from the group consisting of thermal
stability and alkaline stability wherein said
precursor carbonyl hydrolase is selected from the
group consisting of naturally occurring carbonyl
10 hydrolases and recombinant carbonyl hydrolases and
said carbonyl hydrolase mutant amino acid sequence is
derived by a method selected from the group consisting
of the substitution, deletion and insertion of at
least one amino acid in said amino acid sequence of
15 said precursor carbonyl hydrolase.

2. A carbonyl hydrolase mutant having at least one
property which is substantially different from the
same property of a precursor carbonyl hydrolase from
20 which the amino acid sequence of said carbonyl
hydrolase mutant is derived, said property being
selected from the group consisting of oxidative
stability, substrate specificity, catalytic activity,
thermal stability, alkaline stability and pH activity
25 profile wherein said precursor carbonyl hydrolase is
selected from the group consisting of naturally
occurring carbonyl hydrolases and recombinant carbonyl
hydrolases and said carbonyl hydrolase mutant amino
acid sequence is derived by a method selected from the
30 group consisting of deletion and insertion of at least
one amino acid in said amino acid sequence of said
precursor carbonyl hydrolase and substitution of more
than one amino acid residue of said amino acid
sequence of said precursor carbonyl hydrolase.
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3. A carbonyl hydrolase mutant derived by the replacement of at least one amino acid residue of a precursor carbonyl hydrolase with a different amino acid, said one amino acid residue being selected from the group of amino acid residues of Bacillus amyloliquefaciens subtilisin consisting of Tyr21, Thr22, Ser24, Ser33, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Tyr104, Ile107, Gly110, Met124, Asn155, Glu156, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, His67, Leu126, Leu135, Gly97, Asp99, Ser101, Gly102, Glu103, Leu126, Gly127, Gly128, Pro129, Tyr214, Gly215, and equivalent amino acid residues in other precursor carbonyl hydrolases.

4. A carbonyl hydrolase mutant having an amino acid sequence derived from the amino acid sequence of a precursor carbonyl hydrolase by the substitution of a different amino acid for more than one amino acid residue of said amino acid sequence of said precursor carbonyl hydrolase, said amino acid residues being selected from the group of amino acid residues of Bacillus amyloliquefaciens subtilisin consisting of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val95, Leu96, Tyr104, Ile107, Gly110, Met124, Ala152, Asn-155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu126, Leu135, Gly97, Asp99, Ser101, Gly102, Glu103, Leu126, Gly127, Gly128, Pro129, Tyr214, Gly215, and equivalent amino acid residues in other precursor carbonyl hydrolases.

5. The mutant of Claim 4 wherein said combinations are selected from the group consisting of Thr22/Ser87, Ser24/Ser87, Ala45/Ala48, Ser49/Lys94, Ser49/Val195, Met50/Val195, Met50/Gly110, Met50/Met124, Met50/Met222, Met124/Met222, Glu156/Gly166, Glu156/Gly169, Gly166/Met222, Gly169/Met222, Tyr21/Thr22, Met50/Met124/Met222, Tyr21/Thr22/Ser87, Met50/Glu156/Gly166/Tyr217, Met50/Glu156/Tyr217, Glu156/Gly169/Tyr217, Ile170/Lys213, Ser204/Lys213, Met50/Ile107/Lys213 and Ser24/Met50/Ile107/Glu156/Gly166/Gly169/Ser204/Lys213/Gly215/Tyr217.

6. A carbonyl hydrolase mutant derived by the replacement of at least one amino acid residue of a precursor carbonyl hydrolase with a different amino acid, said one amino acid residue being selected from the group of amino acid residues of of Bacillus amyloliquefaciens subtilisin consisting of Tyr21, Thr22, Ser24, Asp32, Ser33, Asp36, Ala45, Gly46, Ala48, Ser49, Met50, Asn77, Ser87, Lys94, Val195, Leu96, Tyr104, Ile107, Gly110, Met124, Ala152, Asn-155, Glu156, Gly166, Gly169, Lys170, Tyr171, Pro172, Phe189, Asp197, Met199, Ser204, Lys213, Tyr217, Ser221, Met222, His67, Leu126, Leu135, Gly97, Asp99, Ser101, Gly102, Glu103, Leu126, Gly127, Gly128, Pro129, Tyr214, Gly215, and equivalent amino acid residues in other precursor carbonyl hydrolases, wherein said at least one amino acid residue of said precursor carbonyl hydrolase is replaced with the amino acid residues listed in TABLE I and TABLE II herein.

7. The mutant of Claim 6 wherein the amino acid replacing said at least one amino acid residue in said precursor carbonyl hydrolase is selected from the replacement amino acids listed in TABLE I herein.

8. Mutant DNA sequence encoding the mutant of claims
1 through 7.

9. Expression vector containing the mutant DNA
sequence of claim 8.

10. Host cell transformed with the expression vector
of Claim 9.

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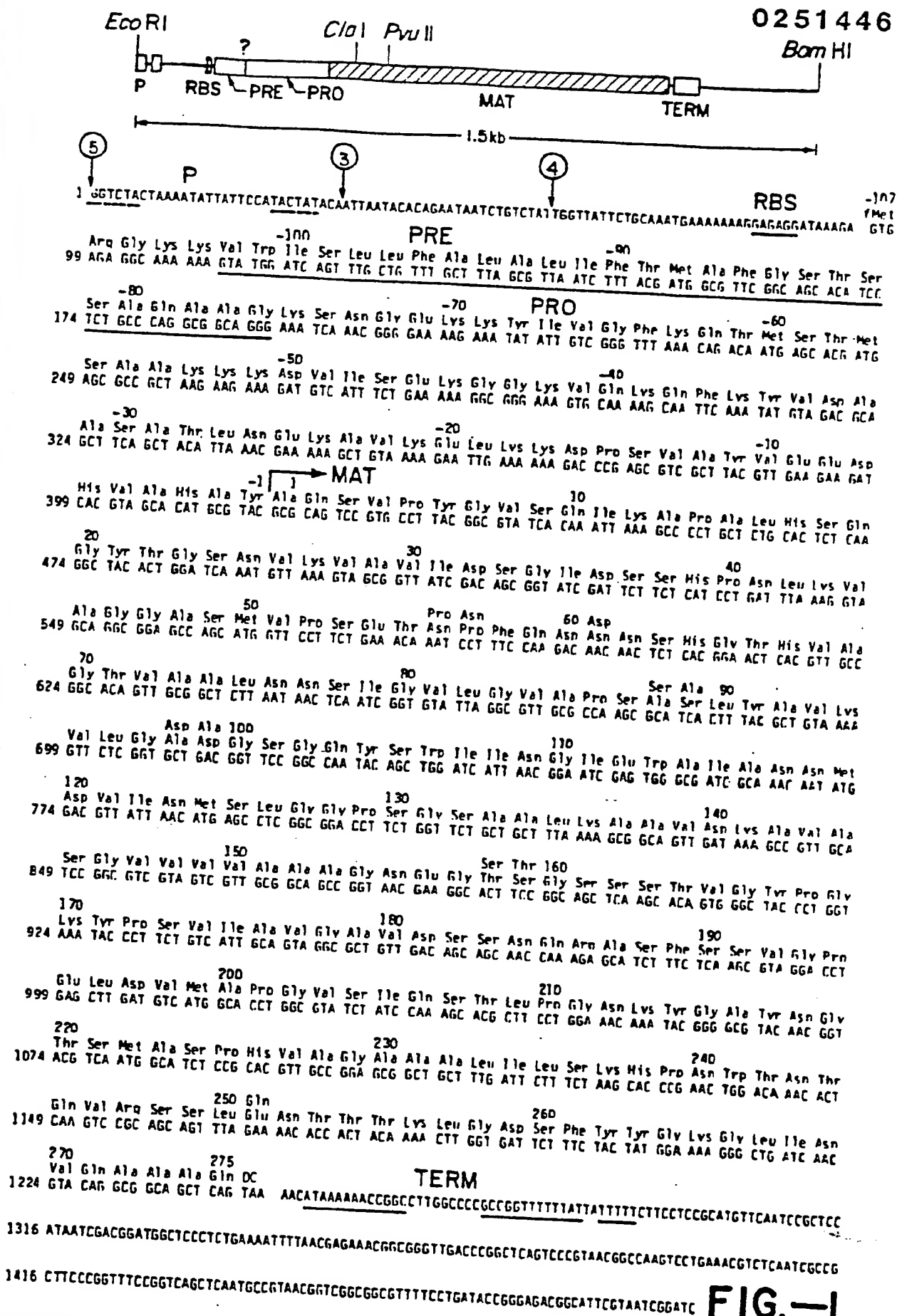


FIG.-1

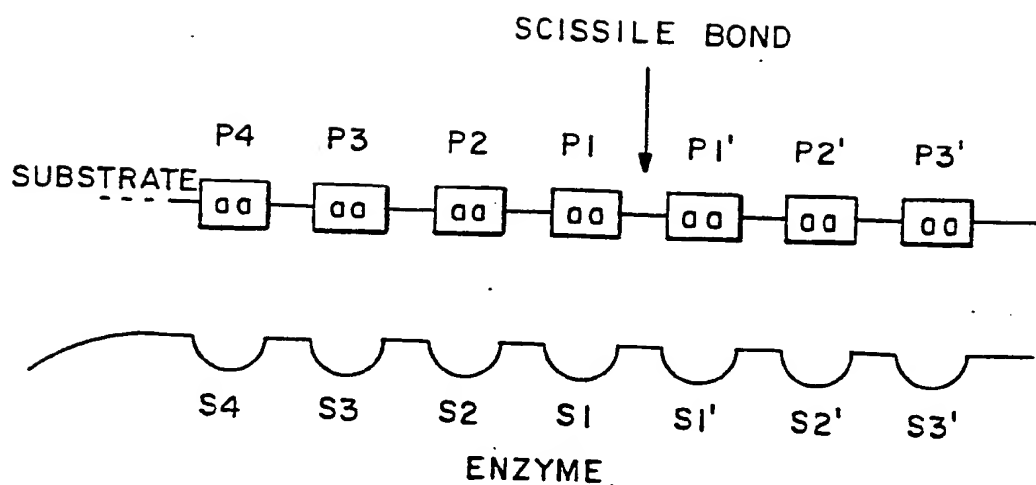


FIG.-2

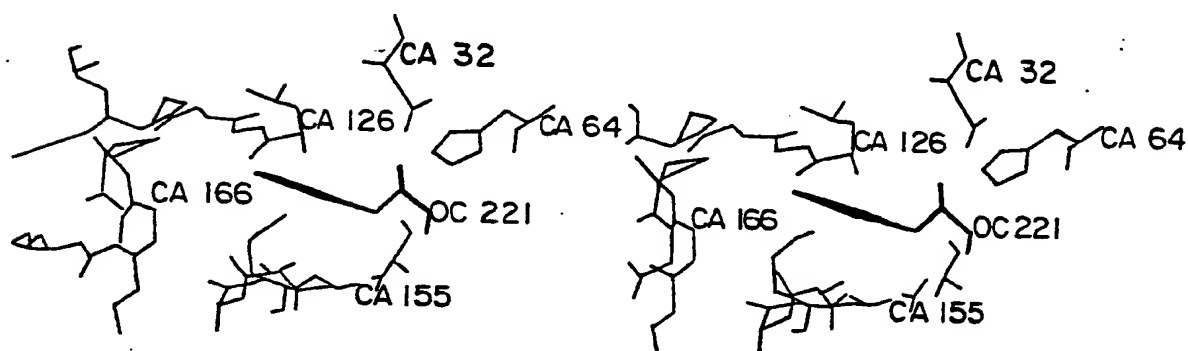


FIG.-3

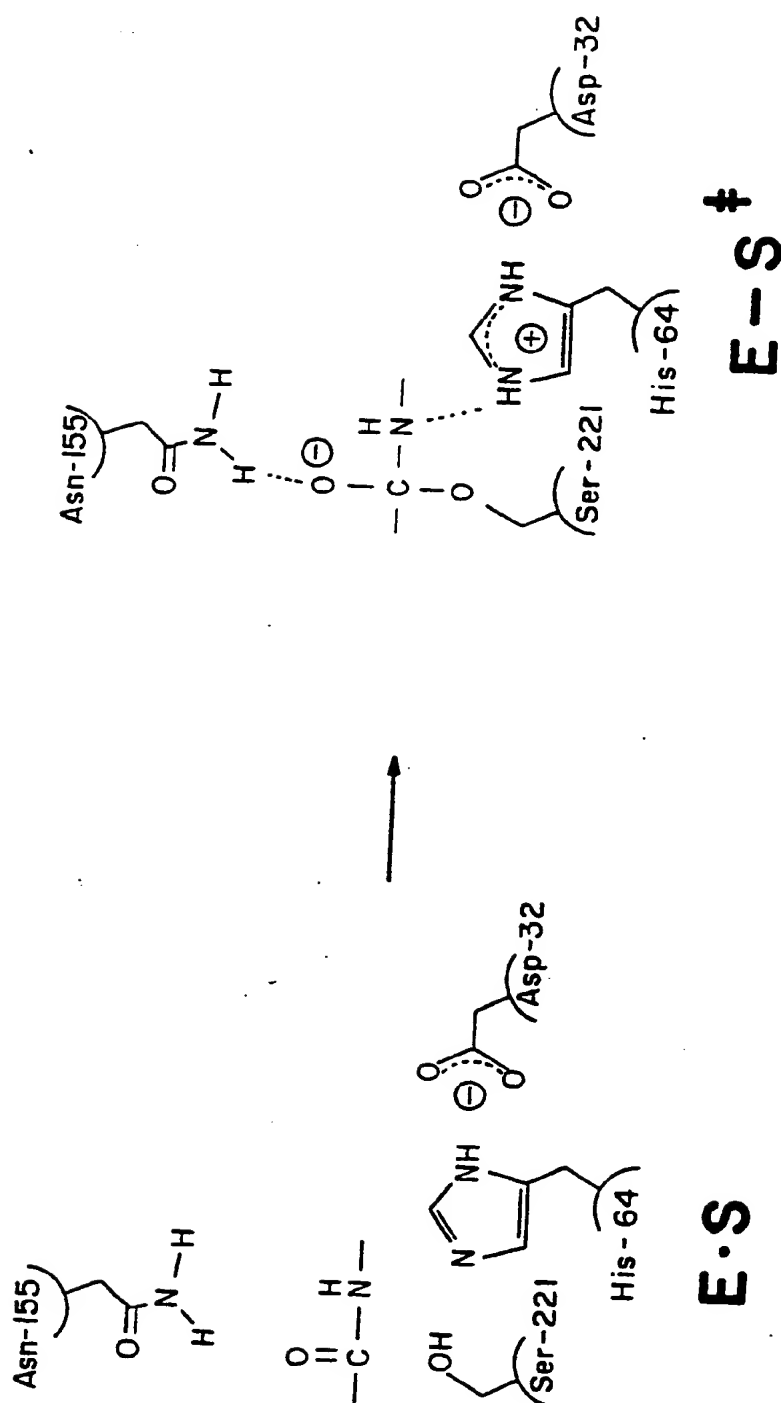


FIG.-4

Homology of *Bacillus* proteases

1. *Bacillus amyloliquefaciens*
2. *Bacillus subtilis* var. 115B
3. *Bacillus licheniformis* (carlsbergensis)

1	A	Q	S	V	P	Y	G	V	S	10	I	K	A	P	A	L	H	S	Q	20	
A	Q	S	V	P	Y	G	G	I	S	Q	I	K	A	P	A	L	H	S	Q	6	
A	Q	T	V	P	Y	G	G	I	S	Q	I	K	A	P	A	L	H	S	Q	6	
21	Y	T	G	S	N	V	K	V	A	30	V	I	D	S	G	I	D	S	S	H	40
Y	T	G	S	N	V	K	K	V	A	V	I	D	S	G	G	I	D	S	S	H	P
F	K	G	A	N	V	K	V	V	A	V	I	D	S	G	G	I	D	S	S	H	P
41	D	L	K	V	A	G	G	A	S	50	H	V	P	S	E	T	N	P	F	Q	60
D	L	N	V	R	G	G	A	S	S	H	V	P	S	E	T	N	P	F	Q	D	D
D	L	N	V	V	G	G	A	S	S	F	V	A	G	E	A	N	P	Y	T	Q	D
61	N	S	H	G	T	H	V	A	70	G	T	V	A	A	L	N	N	S	I	80	
G	S	S	H	G	T	H	V	A	G	G	T	V	A	A	L	N	N	S	I	6	
G	S	S	H	G	T	H	V	A	G	G	T	V	A	A	L	N	N	S	I	6	
81	V	L	G	V	A	P	S	A	S	90	L	Y	A	V	K	V	L	G	A	D	100
V	L	G	V	A	P	S	S	A	S	L	Y	A	V	K	V	L	G	A	D	6	
V	L	G	V	A	P	S	S	A	S	L	Y	A	V	K	V	L	G	A	D	6	
121	S	G	Q	Y	S	W	I	I	N	110	G	I	E	W	A	I	A	N	N	M	120
S	G	Q	Y	S	W	I	I	I	N	G	I	E	W	A	I	A	N	N	M	D	D
S	G	S	Y	S	G	I	I	I	S	G	I	E	W	A	I	A	N	N	M	D	D

FIG.—5A-1

121	V	I	N	M	S	L	6	6	P	130	S	6	S	A	A	L	K	A	A	V	140	D
	V	I	N	M	S	L	6	6	P		T	6	S	T	A	L	K	T	A	V		D
	V	I	N	M	S	L	6	6	A		S	6	S	T	A	L	K	Q	A	V		D
141	K	A	V	A	S	6	V	V	V	150	V	A	A	A	6	N	E	6	T	S	160	6
	K	A	V	A	S	6	V	V	V		A	A	A	A	6	N	E	6	S	S		6
	N	A	Y	A	R	6	V	V	V		V	A	A	A	6	N	S	6	N	S		6
161	S	S	T	V	6	Y	P	6	K	170	Y	P	S	V	I	A	V	6	A	180	V	
	S	T	S	T	6	Y	P	A		K	Y	P	S	T	I	A	V	6	A		V	
	S	T	N	T	6	Y	P	A		K	Y	D	S	V	I	A	V	6	A		V	
181	D	S	S	N	Q	R	A	S	F	190	S	S	V	6	P	E	L	D	V	200	A	
	N	S	S	N	Q	R	A	S	F		S	S	V	6	S	E	L	D	V		A	
	D	S	S	N	N	R	A	S	F		S	S	V	6	A	E	L	D	V		A	
201	P	6	V	S	I	Q	S	T	L	210	P	6	N	K	Y	6	A	Y	N	220	T	
	P	6	V	S	I	Q	S	T	L		P	6	N	K	Y	6	A	Y	N		T	
	P	6	A	6	V	Y	S	T	Y		P	T	N	T	Y	A	T	L	N		T	
221	S	M	A	S	P	H	V	A	6	230	A	A	A	L	I	L	S	K	H	240	N	
	S	M	A	S	P	H	V	A	6		A	A	A	L	I	L	S	K	H		N	
	S	M	A	S	P	H	V	A	6		A	A	A	L	I	L	S	K	H		N	
241	W	T	N	T	Q	V	R	S	S	250	L	E	N	T	T	T	K	L	6	260	S	
	W	T	N	T	Q	V	R	S	S		L	E	N	T	T	T	K	L	6		S	
	L	S	A	S	Q	V	R	N	R		L	S	S	T	A	T	Y	L	6		S	
261	F	Y	Y	6	K	6	L	I	N	270	V	Q	A	A	A	Q						
	F	Y	Y	6	K	6	L	I	N		V	Q	A	A	A	Q						
	F	Y	Y	6	K	6	L	I	N		V	E	A	A	A	Q						

FIG.—5A—2

FIG.—5B—1

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A A A G N E S T S 150
 A A A G N A G N T A P N Y P S 170
 Y P S U I A U G A 180
 Y S N A I A U A S T D D S N D N K A S S F S 190
 S U G P E L D U H A 200
 T Y G S U U D U A A P G U S I D S T L 210
 G N K Y S A Y N G T S H A S P H U A G 230
 T S T Y A S L S G T S H A T P H U A G U
 A A L I L S K H P N 240
 A G L L A S D B R S . . A S N I R A A 250
 E N T T T K . L S D S 260
 E N T A D K I S G T S F Y Y G K G L I N
 270
 U G A A A D
 A Y K A U D Y

FIG.—5B-2

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FIG.—5C

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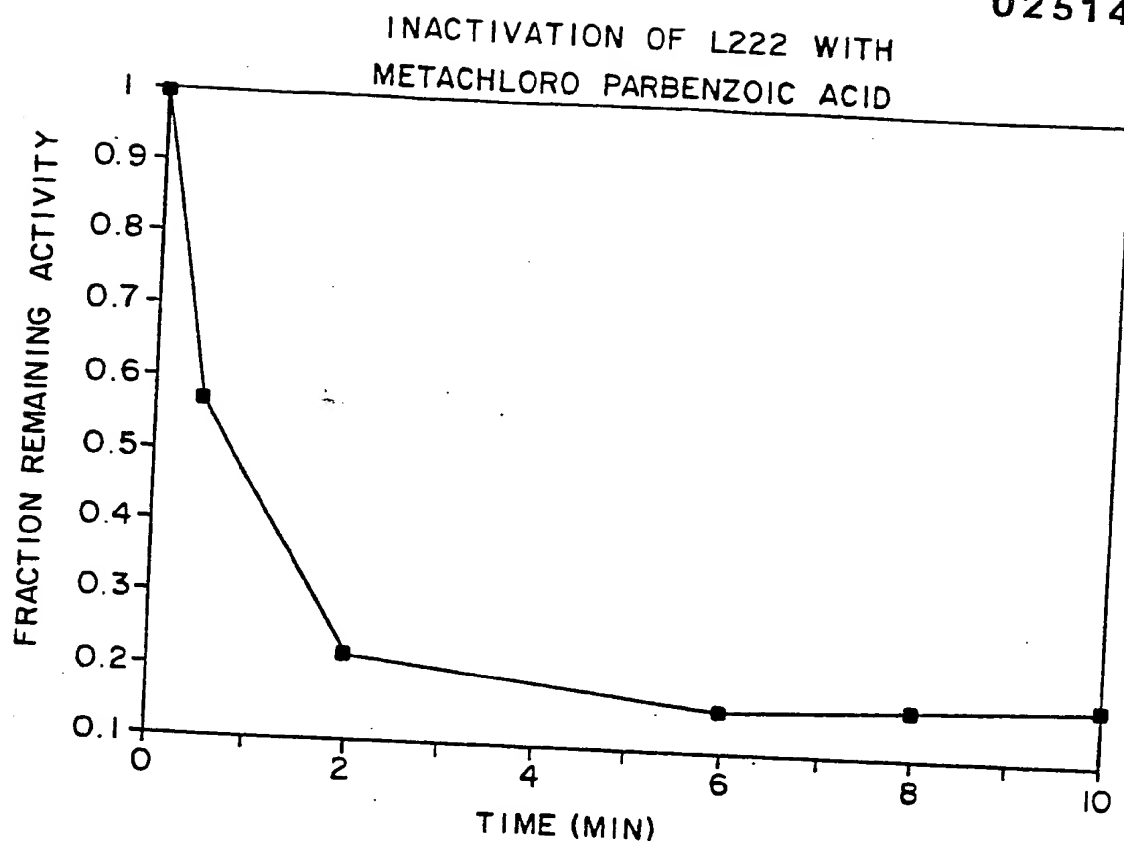


FIG.-6A

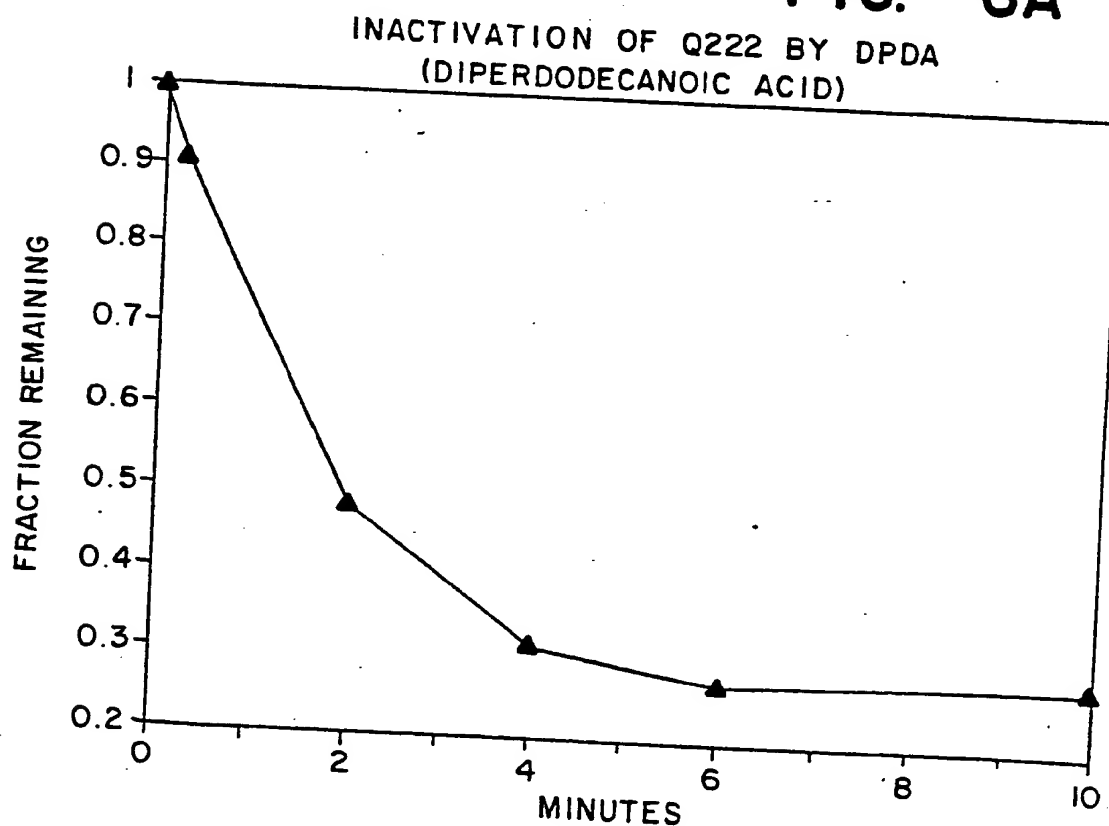


FIG.-6B

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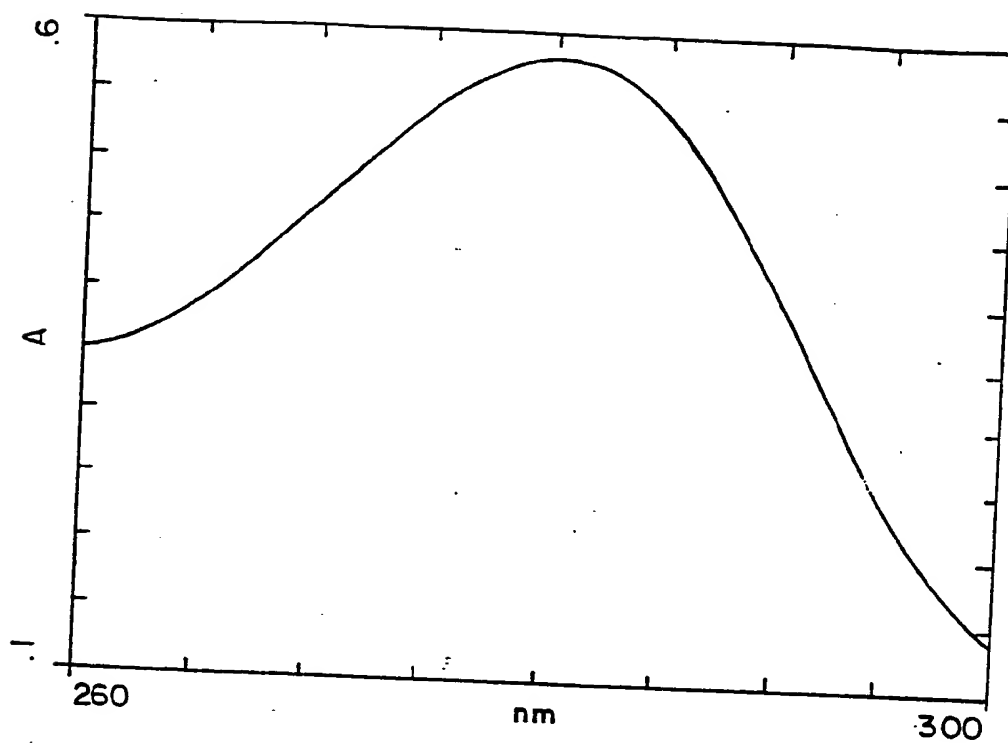


FIG. -7A

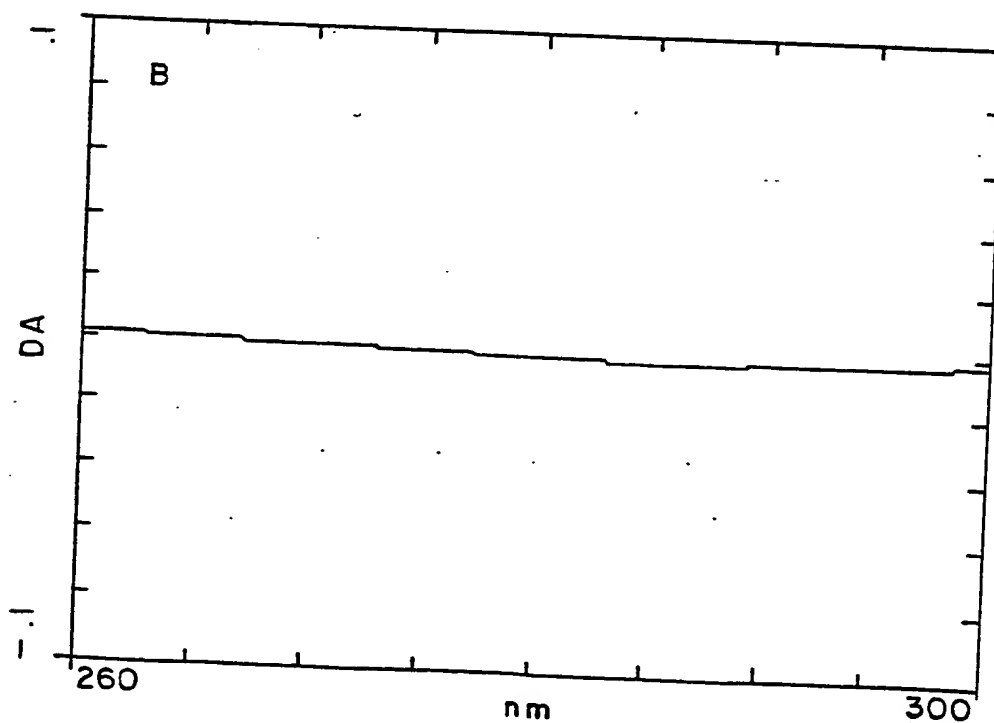


FIG. -7B

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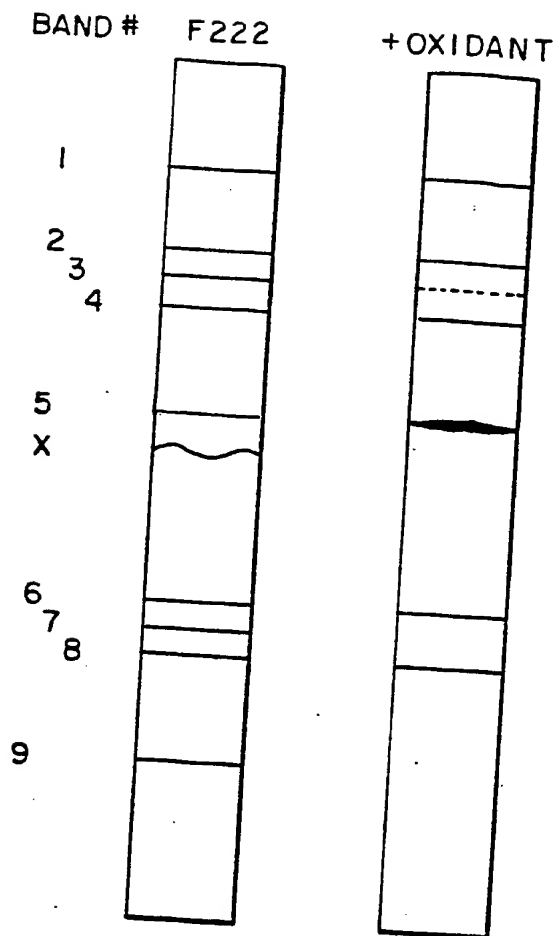


FIG.- 8

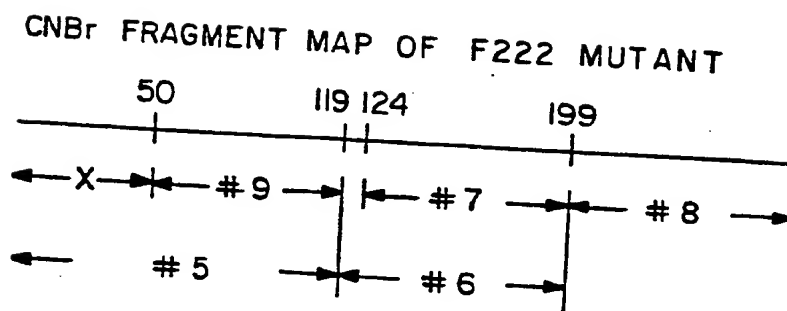


FIG.-9

FIG. -10

8. Mutants made:

V45, P45, V45/P48, E46, E48, V48, C49, C50, F50

FIG. -10

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1. Codon number:

2. Wild type amino acid sequence:

3. Wild type DNA sequence:

43 45

Lys-Val-Ala-Gly-Gly-Ala-Ser-Met-Val-Pro-Ser
5'-AAG-GTA-GCA-GGC-GGA-GCC-AGC-ATG-GTT-CCT-TCT
TTC-CAT-CGT-CCG-CCT-CGG-TCG-TAC-CAA-GGA-AGA-5'

4. p450:

5' - AAG-GCC-T-
TTC-CGG-A-
Su1

5. p450 cut with StuI/Kpn I

5'-AAG-G
TTC-Cp

6. Cut pΔ50 ligated with cassettes:

5' -AAG-GTA-GCA-GGC-GGA-GCC-AGC-ATG-GTA-CCT-TCT
TCC-CAT-CGT-CCG-CCT-CGG-TCG-TAC-CAT-GGA-AGA-5' *

7. Mutagenesis primer for pΔ50:

5'-CT-GAT-TTA-AAG-GCC-TGC-ATG-GTA-CCT-TCT-GA

8. Mutants made:

1. Codon number: 117 120 124 126 130
2. Wild type amino acid sequence: Asn-Asn-Met-Asp-Val-Ile-Asn-Met-Ser-Leu-Gly-Gly-Pro-Ser
3. Wild type DNA sequence: 5'-AAC-AAT-ATG-GAC-GTT-ATT-AAC-ATG-AGC-CTC-GGC-GGA-CCT-TCT
TTG-TTA-TAC-CTG-CAA-TAA-TTG-TAC-TCG-GAG-CCG-CCG-GGA-AGA-5'
4. pΔ124:

* * * *
 5'-AAC-AAT-ATG-GAT-ATC-----C-GGC-GGC-CCT-TCT
 TTG-TTA-TAC-CTA-TAG-----G-CCC-CCG-GGA-AGA-5'

Eco RV Apa I
5. pΔ124 cut with Eco RV and Apa I

*
 5'-AAC-AAT-ATG-GAT
 TTG-TTA-TAC-CTAP

 *
 PCT-TCT
 CCG-GGA-AGA-5'
6. Cut pΔ124 ligated with cassettes:

*
 5'-AAC-AAT-ATG-GAT-GTT-ATT-AAC-ATG-AGC-CTC-GGC-GGC-CCT-TCT
 TTG-TTA-TAC-CTA-CAA-TAA-TTG-TAC-TCG-GAG-CCG-CCG-GGA-AGA-5'
7. Mutagenesis primer for pΔ124::

* * * *
 5'-AAC-AAT-ATG-GAT-ATC-C-GGG-GGC-CCT-TCT-GGT-TC-3'
8. Mutants made: I 124, L 124 AND C 126

FIG.—II

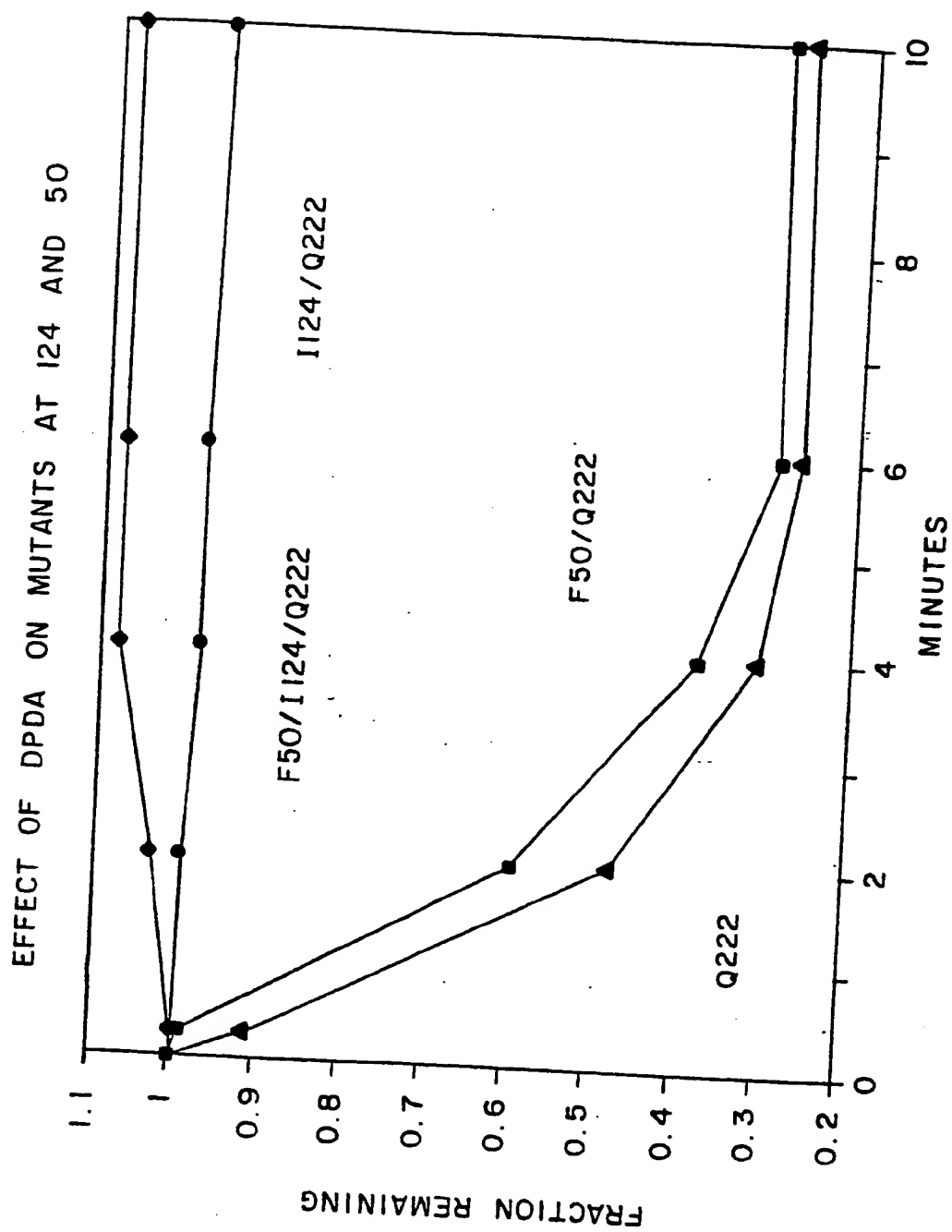


FIG.-12

Wild type amino acid sequence:

166

Thr Ser Gly Ser Ser Ser Thr Val Gly Tyr Pro Gly

5'-ACT TCC GGC AGC TCA AGC ACA GTG GGC TAC CCT GGT-3'

3'-TGA AGG CCG TCG AGT TCG TGT CAC CCG ATG GGA CCA-5'

1. Wild type DNA sequence:

2. pΔ166 DNA sequence:

5'-ACT TCC GGG AGC TCA A

3'-TGA AGG CCC TCG AGT T

SacI

C CCG GGT-3'

G GGC CCA-5'

XmaI

3. pΔ166 cut with SacI and XmaI:

5'-ACT TCC GGG AGC T

3'-TGA AGG CCCp

*

PCCG GGT-3'

CA-5'

4. Cut pΔ166 ligated with duplex DNA cassette pools:

5'-ACT TCC GGG AGC TCA AGC ACA GTG NNN TAC CCG GGT-3'

3'-TGA AGG CCC TCG AGT TCG TGT CAC NNN ATG GGC CCA-5'

*

MUTAGENESIS PRIMER 37 MER

5' AA GGC ACT TCC GGG AGC TCA ACC CGG GTA AA TAC CCT 3'

FIG.—13

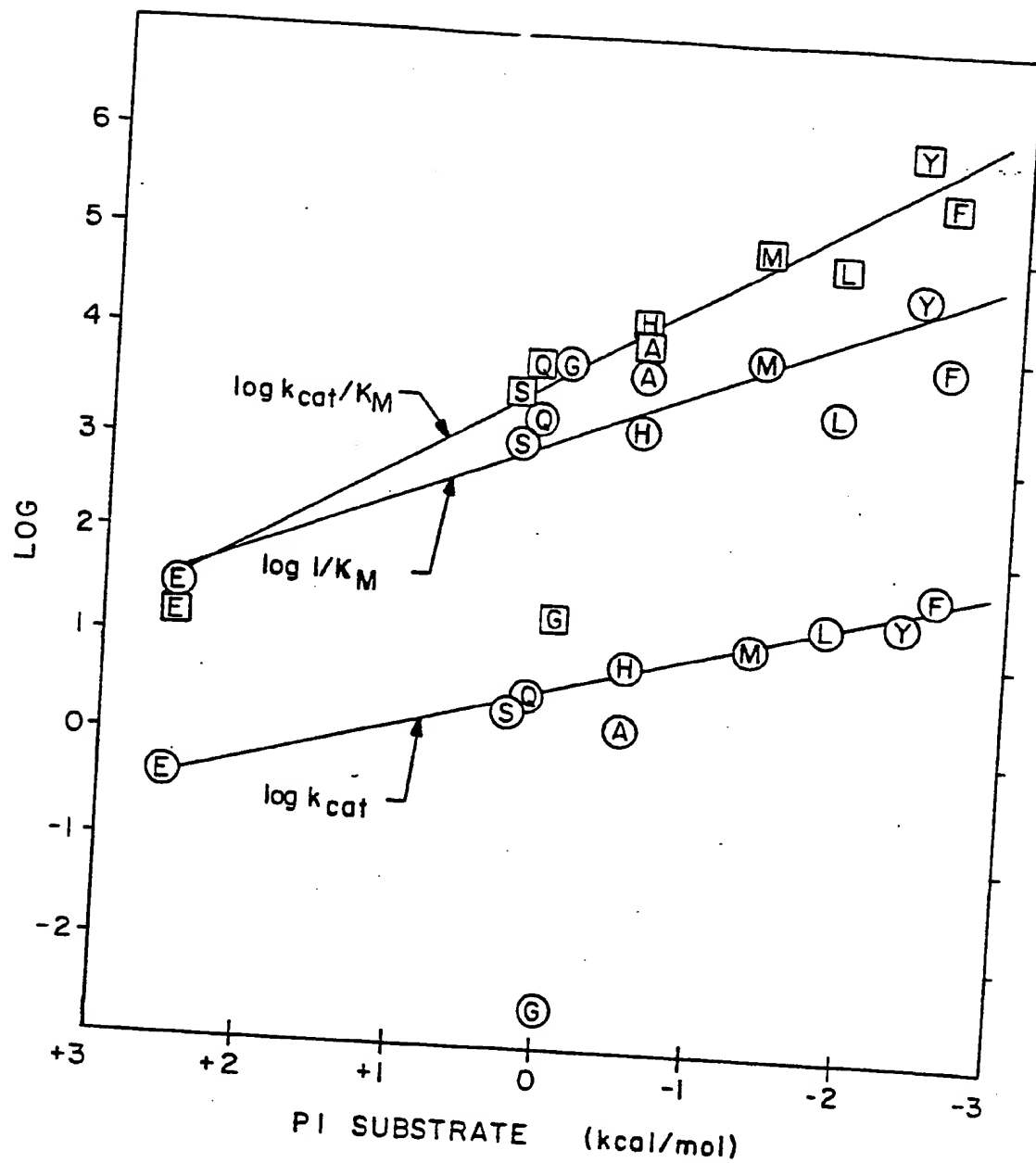


FIG. - 14

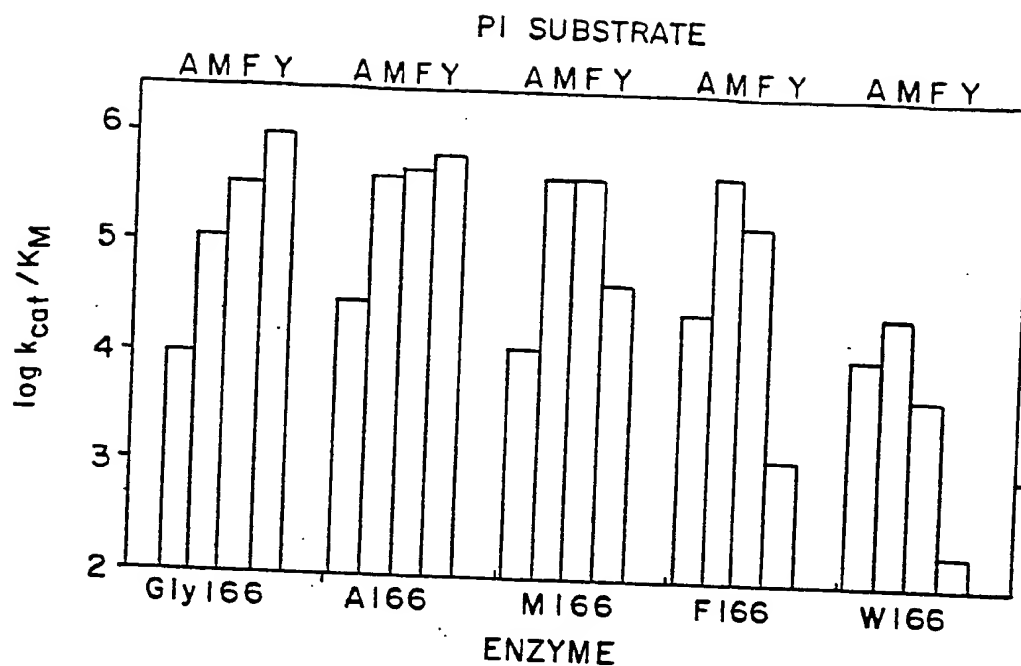


FIG.-15A

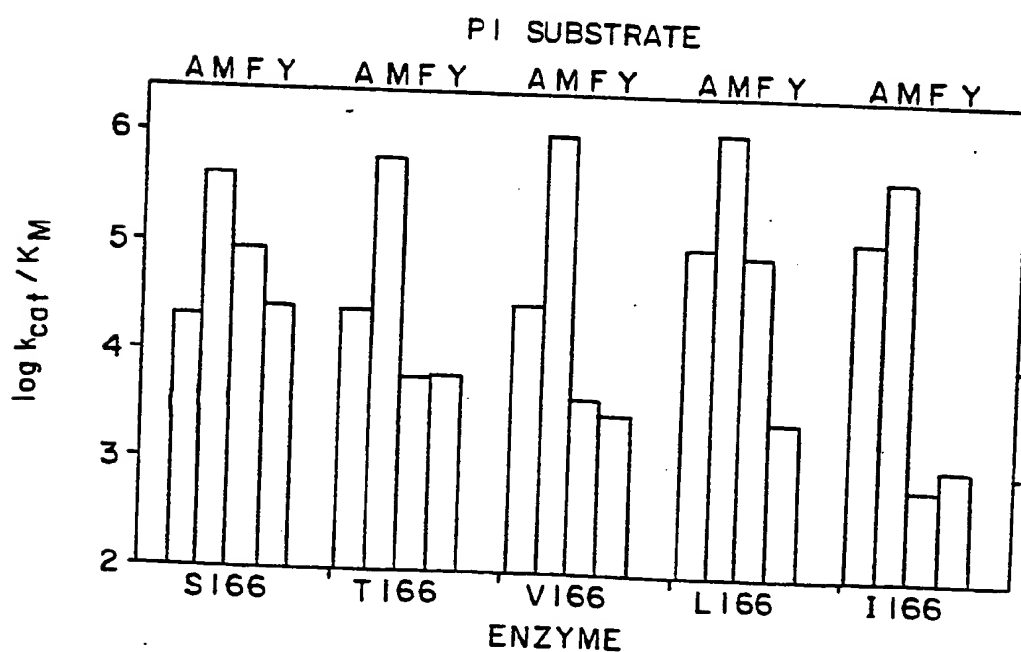


FIG.-15B

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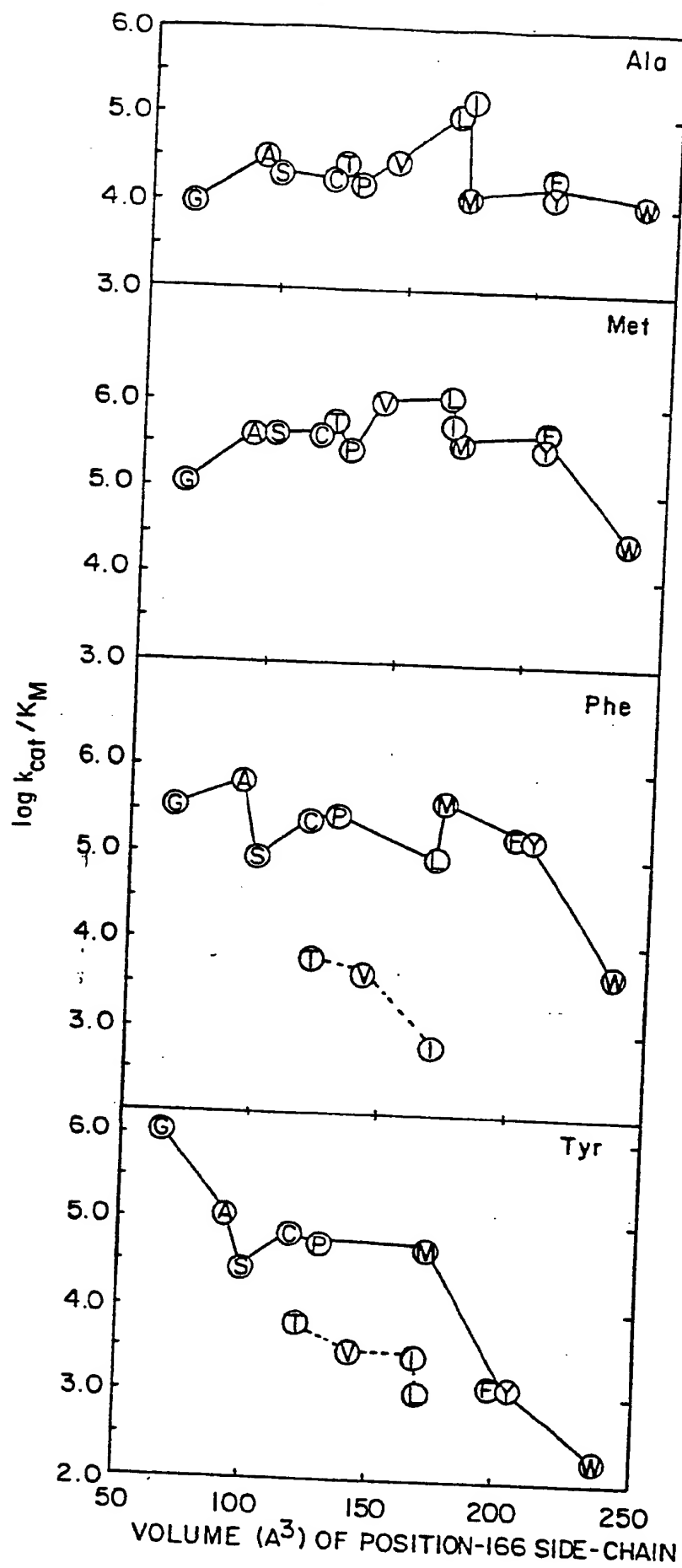


FIG.-16

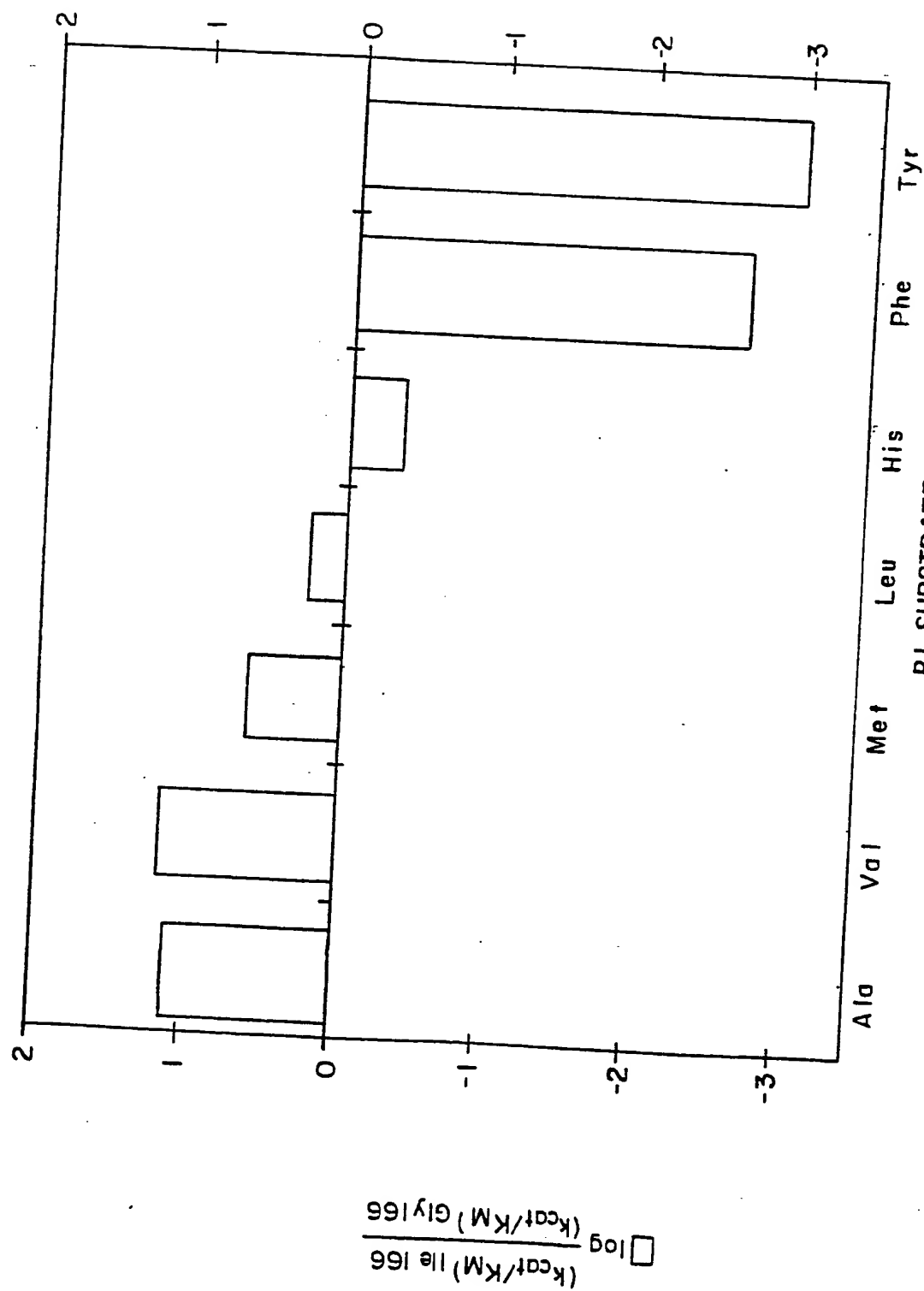


FIG. - 17

0251446

162
SER SER THR VAL GLY TYR PRO GLY 169 173
5' TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT 3'
3' AGT TCG TGT CAC CCG ATG GGA CCA TTT ATG GGA AGA 5'

5' TCA AGC ACA GTG GGC TAC CCT GGT AAA TAC CCT TCT 3'
3' AGT TCG TGT CAC CCG ATG GGA CCA TTT ATG GGA AGA 5'

5' TCA AGC ACA GTC GGG TAC CCT-----GA TAT CCT TCT 3'
3' AGT TCG TGT CAC CCC ATG GGA KPN1
CT ATA GGA AGA 5'
ECORV

5' TAC AGC ACA GTC GGG TAC
3' AGT TCG TGT CAC CCP

5' TAC AGC ACA GTG GGG TAC CCT NNN AAA TAT CCT TGT 3'
3' AGT TCG TGT CAC CCC ATG GGA NNN TTT ATA GGA AGA 5'

5' AAG CAC AGT GGG GTA CCC TGA TAT CCT TCT GTC A 3'

81-18

1. Codon number: 100 104 105 108
2. Wild type amino acid sequence: Gly-Ser-Gly-Gln-Tyr-Ser-Trp-Ile-Ile-
3. Wild type DNA sequence: 5'-GGT-TCC-GGC-CAA-TAC-AGC-TGG-ATC-ATT-3'
Pu II
4. Primer for *Hind* III
Insertion at 104: ****
5'-GGT-TCC-GGC-CAA-GCTT-AGC-TGG-ATC-ATT-3'
Hind III
5. Primers for 104 mutants: ***
5'-----T-TCC-GCC-CAA-NNN-AGC-TGG-ATC-----3'
6. Mutants made:
A, M, L, S, AND H104

FIG.—19

1. Codon number: 148 150 152 155
2. Wild type amino acid sequence: Val-Val-Val-Ala-Ala-Ala-Gly-Asn-Glu
3. Wild type DNA sequence: 5'-GTA-GTC-GTT-GCG-GCA-GCC-GGT-AAC-GAA-3'
4. VI52/PI53 5'-GTA-GTC-GTT-GCG-GTA-CCC-GGT-AAC-GAA-3'

GTA-CCC

* * *
5. S 152: 5'-GTA-GTC-GTT-GCG-AGC-GCC-GGT-AAC-GAA-3'

6. G 152: 5'-GTA-GTC-GTT-GCG-GGC-GCC-GGT-AAC-GAA-3'

**

FIG.-20

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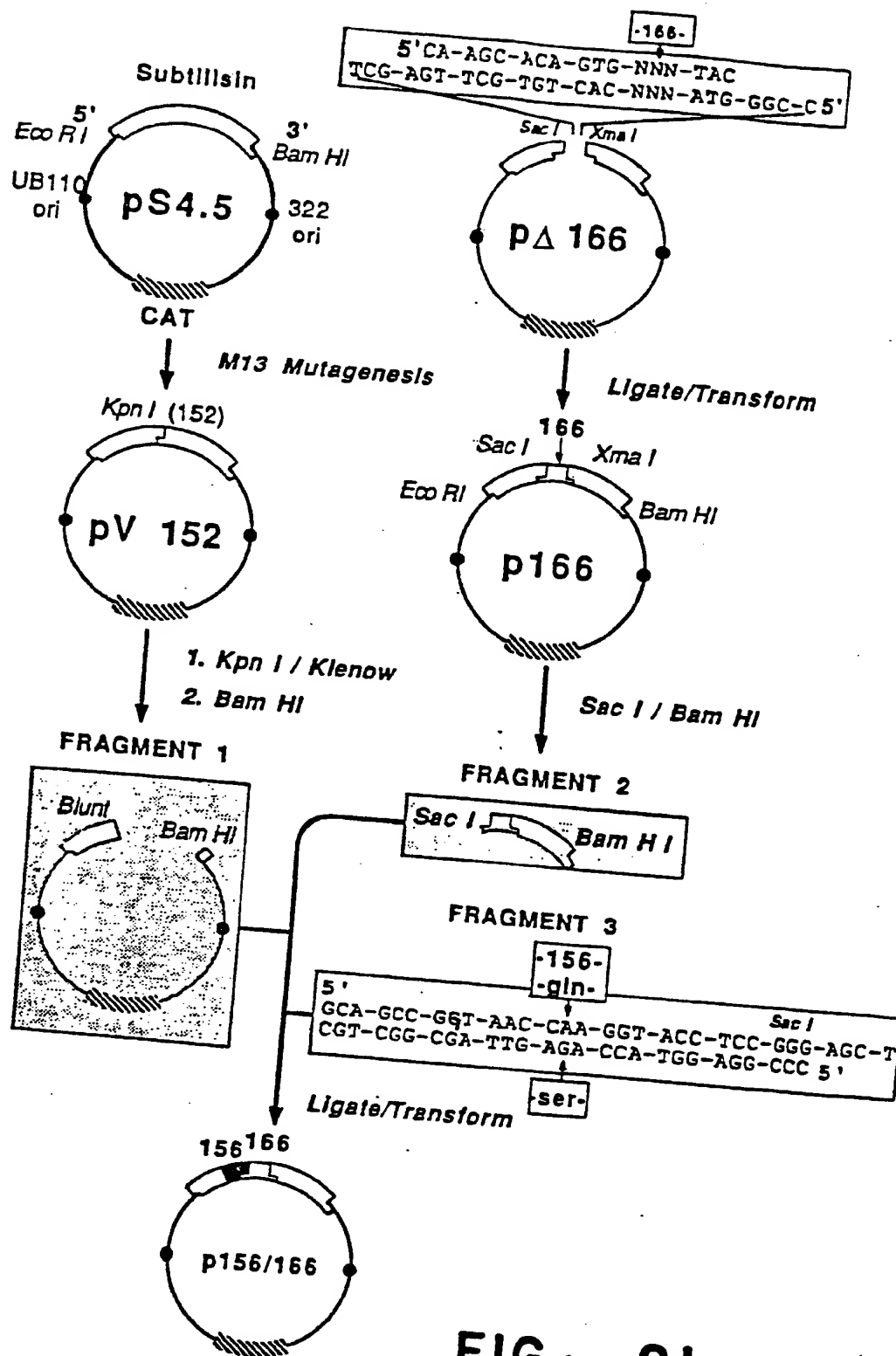


FIG.— 21

1. Codon number: 211 215 217 220
2. Wild type amino acid sequence: Gly-Asn-Lys-Tyr-Gly-Ala-Tyr-Asn-Gly-Thr-Ser-Met-Ala
3. Wild type DNA sequence: 5'-GGA-AAC-AAA-TAC-GGG-GCG-TAC-AAC-GGT-ACG-TCA-ATG-GCA
CCT-TTG-TTT-ATG-CCC-CGC-ATG-TTG-CCA-TGC-AGT-TAC-CGT-5'
4. pΔ217
5'-GGA-AAC-AAA-TAC-GGC-GCC-TAC-----GG-ATA-TCA-ATG-GCA
CCT-TTG-TTT-ATG-CCG-CGG-ATG-----CC-TAT-AGT-TAC-CGT-5'
Nar I Eco RV
5. pΔ217 cut with Nar I and Eco RI
5'-GGA-AAC-AAA-TAC-GG*
CCT-TTG-TTT-ATG-CCG-Gp
* PA-TCA-ATG-GCA
T-AGT-TAC-CGT-5'
6. Cut pΔ217 ligated with cassettes:
5'-GGA-AAC-AAA-TAC-GGC-GCG-NNN-^{***}AAC-GGT-^{*}ACA-TCA-ATG-GCA
CCT-TTG-TTT-ATG-CCG-CGC-NNN-TTG-CCA-TGT-AGT-TAC-CGT-5'
7. Mutagenesis primer for pΔ217:
5'-GA-AAC-AAA-TAC-GGC-GCC-TAC-GGA-TAT-CAA-TGG-CAT-3'
* * *
8. Mutants made: All 19 at 217

FIG.-22

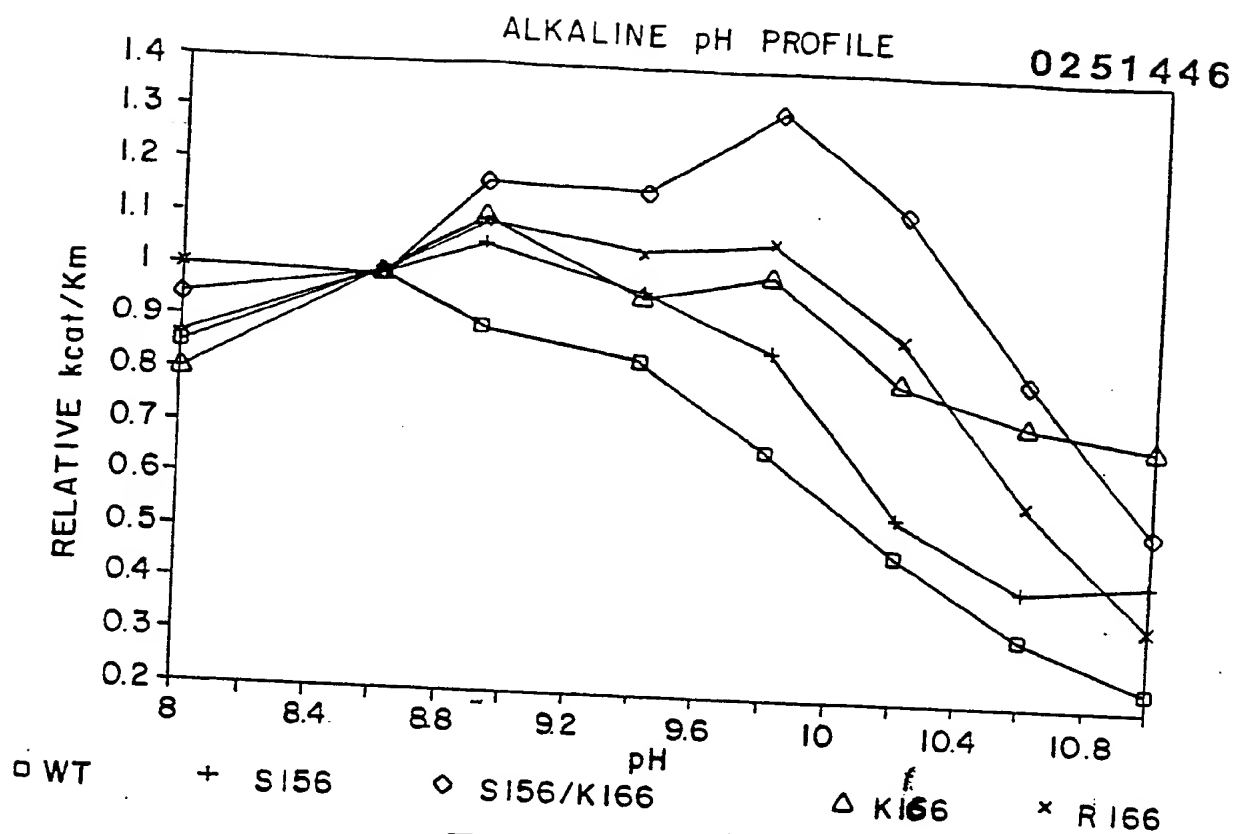


FIG. - 23A

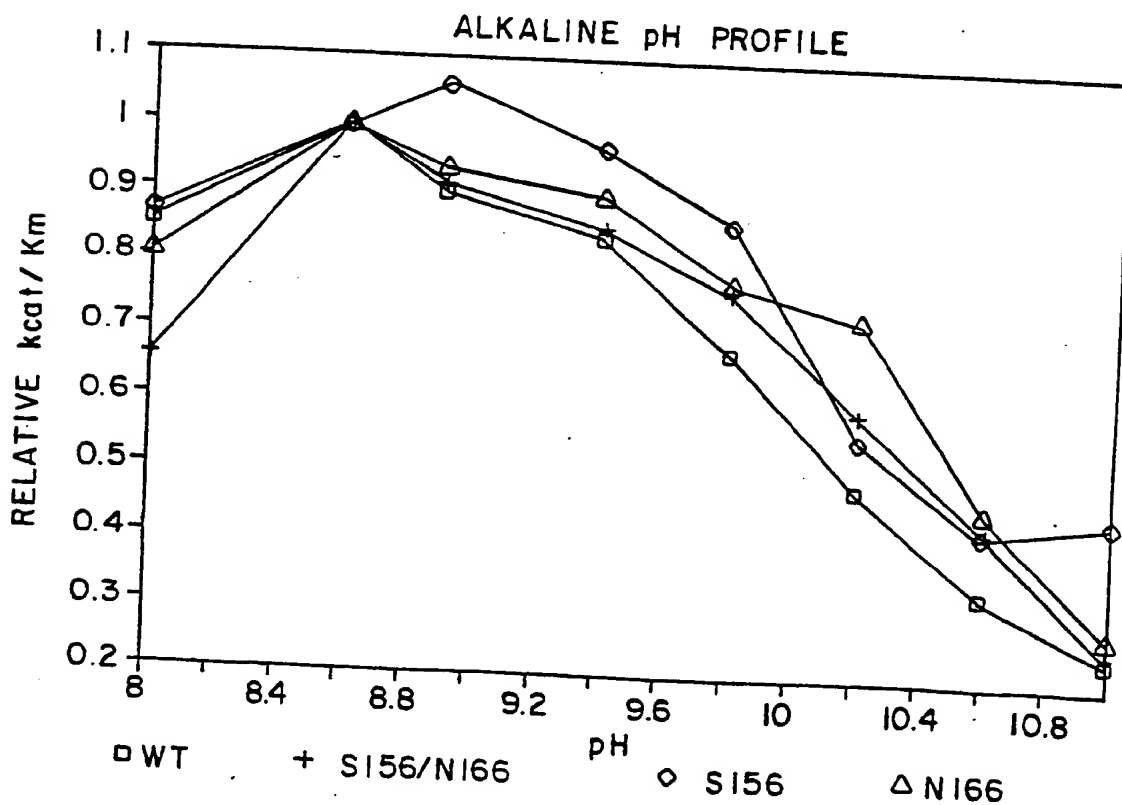


FIG. - 23B

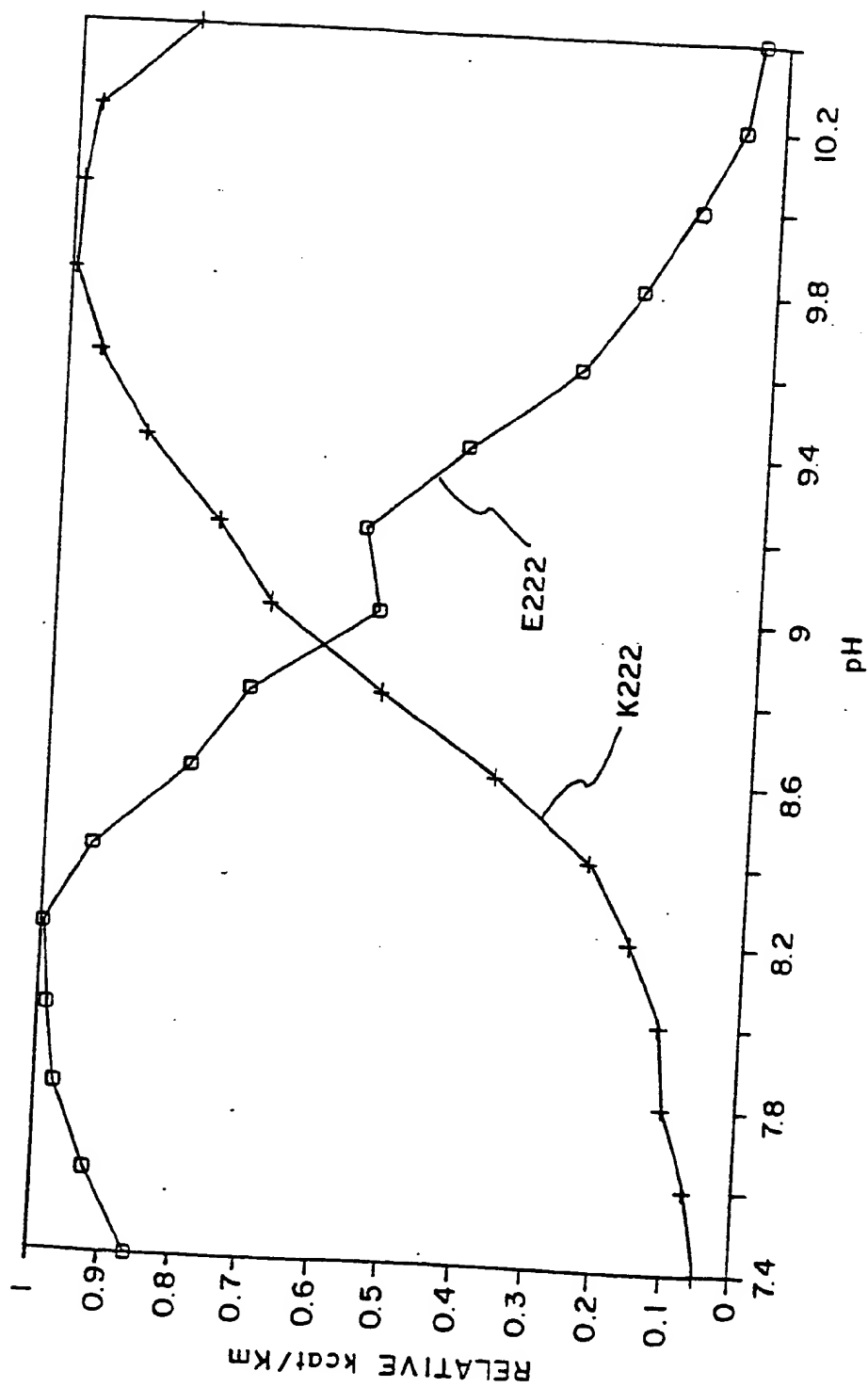


FIG.-24

1. Codon number: 91 95 100
2. Wild type amino acid sequence: Tyr-Ala-Val-Lys-Val-Leu-Gly-Ala-Asp-Gly-Ser
3. Wild type DNA sequence: 5'-TAC-GCT-GTA-AAA-GTT-CTC-GGT-GCT-GAC-GGT-TCC
ATG-CGA-CAT-TTT-CAA-GAG-CCA-CGA-CTG-CCA-AGG-5'
4. pΔ95: 5'-TAC-GCG-T-----CTC-GCT-GCA-GAC-GGT-TCC
ATG-CGC-A-----GAG-CGA-CGT-CTG-CCA-AGG-5'
M_uI Pst I
5. pΔ95 cut with M_uI and Pst I 5'-TA * PGAC-GGT-TCC
ATG-CGCp A-CGT-CTG-CCA-AGG-5'
6. Cut pΔ95 ligated with cassettes: 5'-TAC-GCG-GTA-AAA-GTT-CTC-GGT-GCA-GAC-GGT-TCC
ATG-CGC-CAT-TTT-CAA-GAG-CCA-CGA-CGT-CTG-CCA-AGG-5'
7. Mutagenesis primer for pΔ95: 5'-CA-TCA-CTT-TAC-GCG-T-CTC-GCT-GCA-GAC-GGT-TCC
* * * *
8. Mutants made: C94, C95, D96

FIG.—25

SUBSTRATE SPECIFICITY
pH = 8.60, T = 25

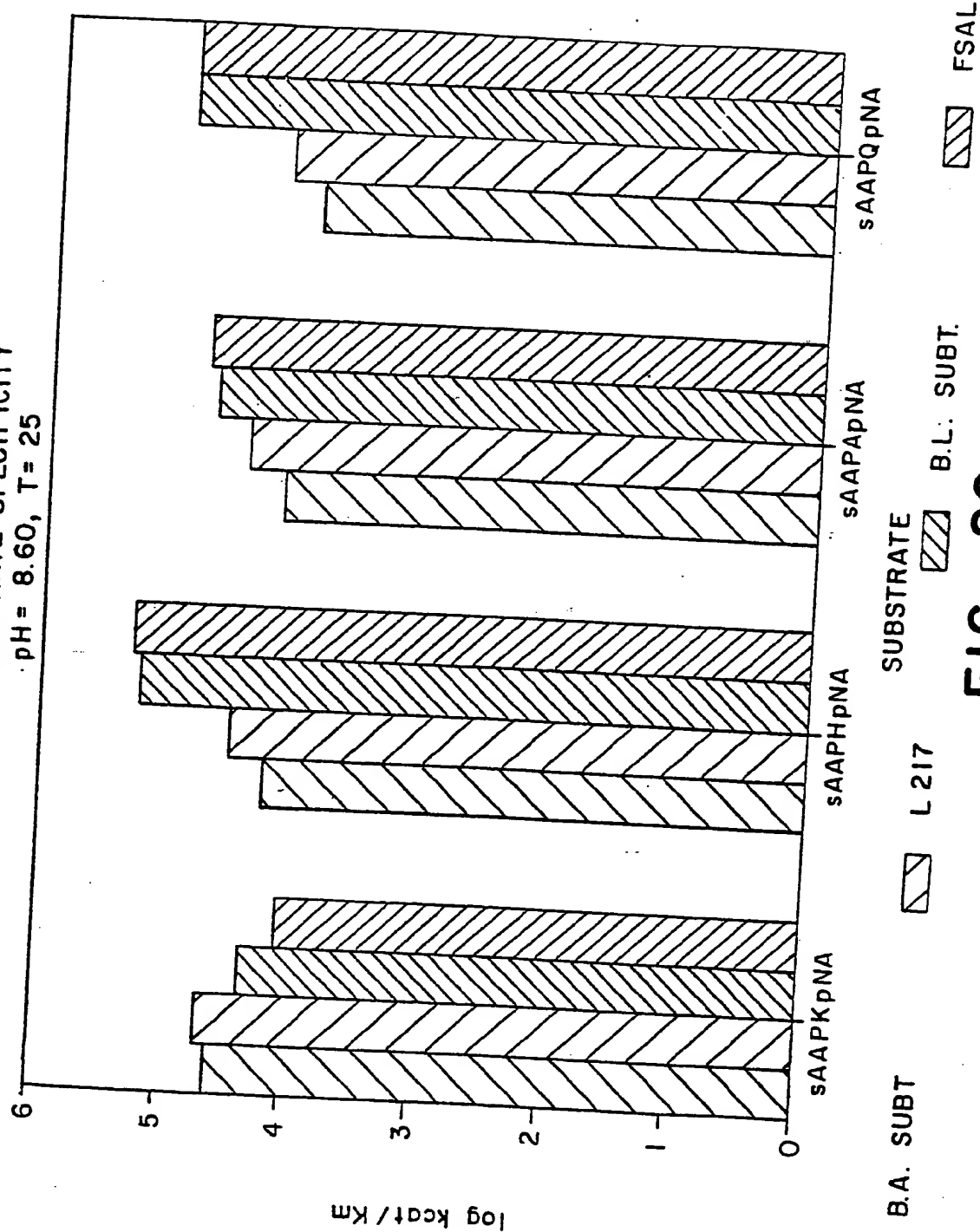


FIG.-26

SUBSTRATE SPECIFICITY
pH = 8.60, T = 25

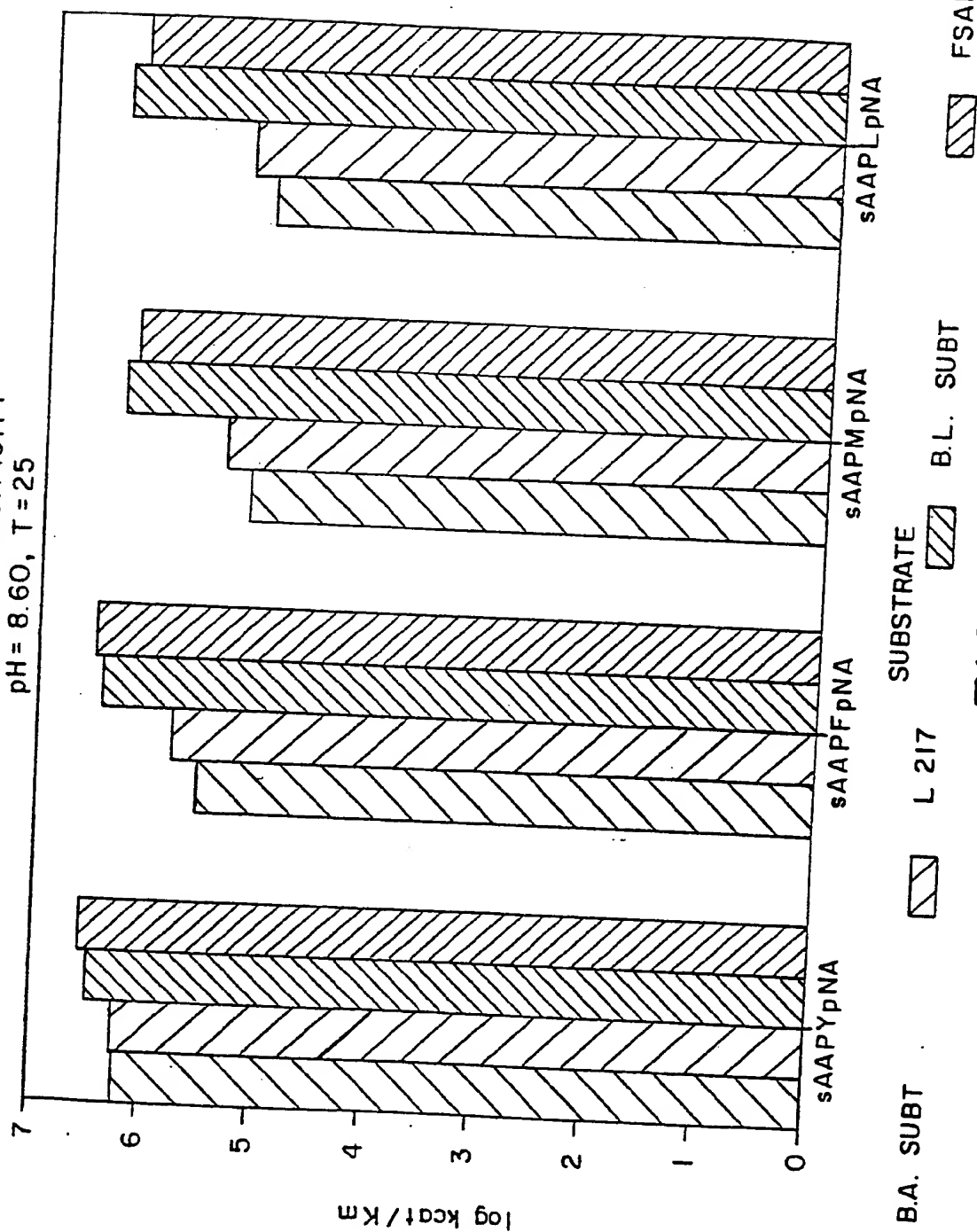


FIG.-27

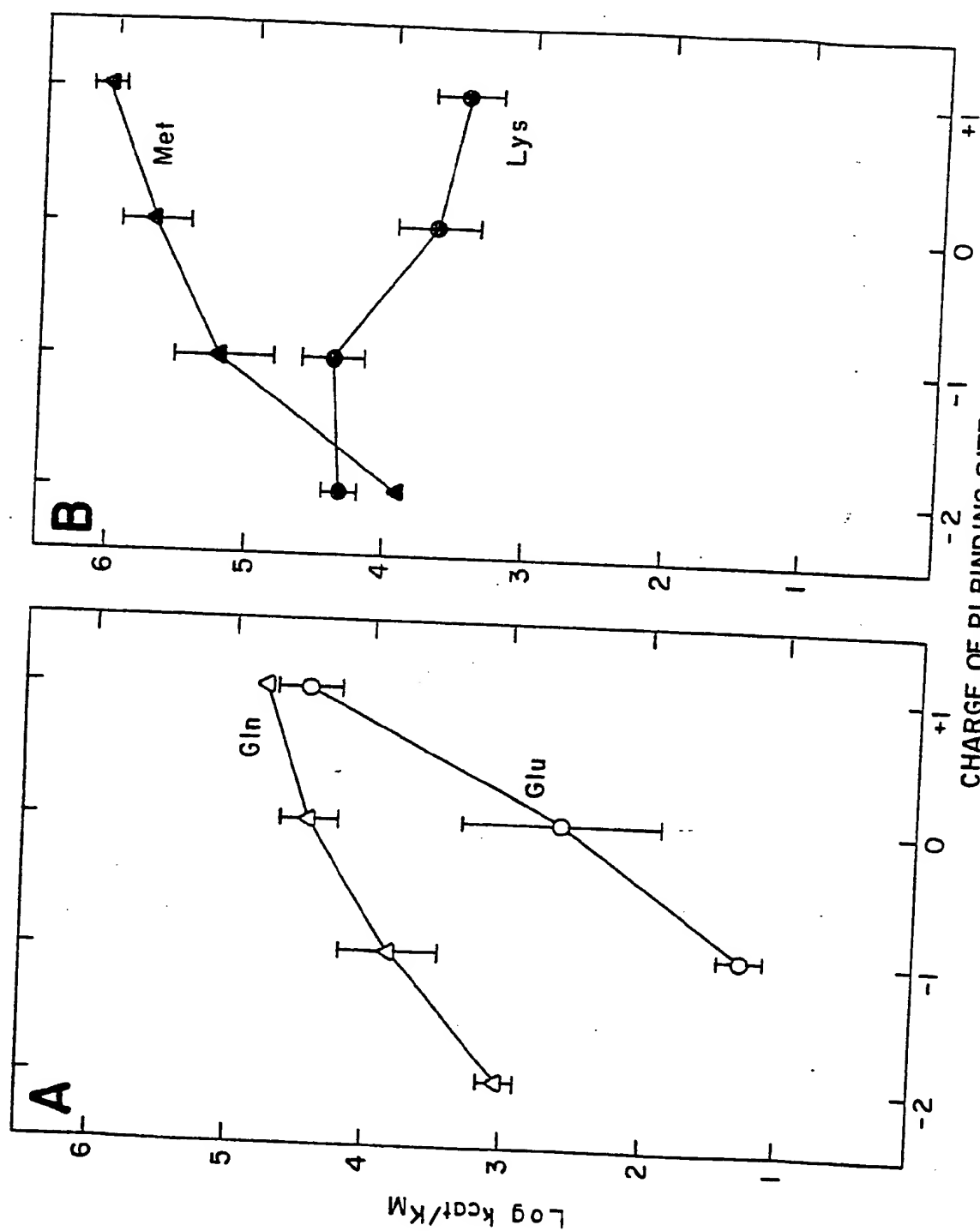


FIG.-28

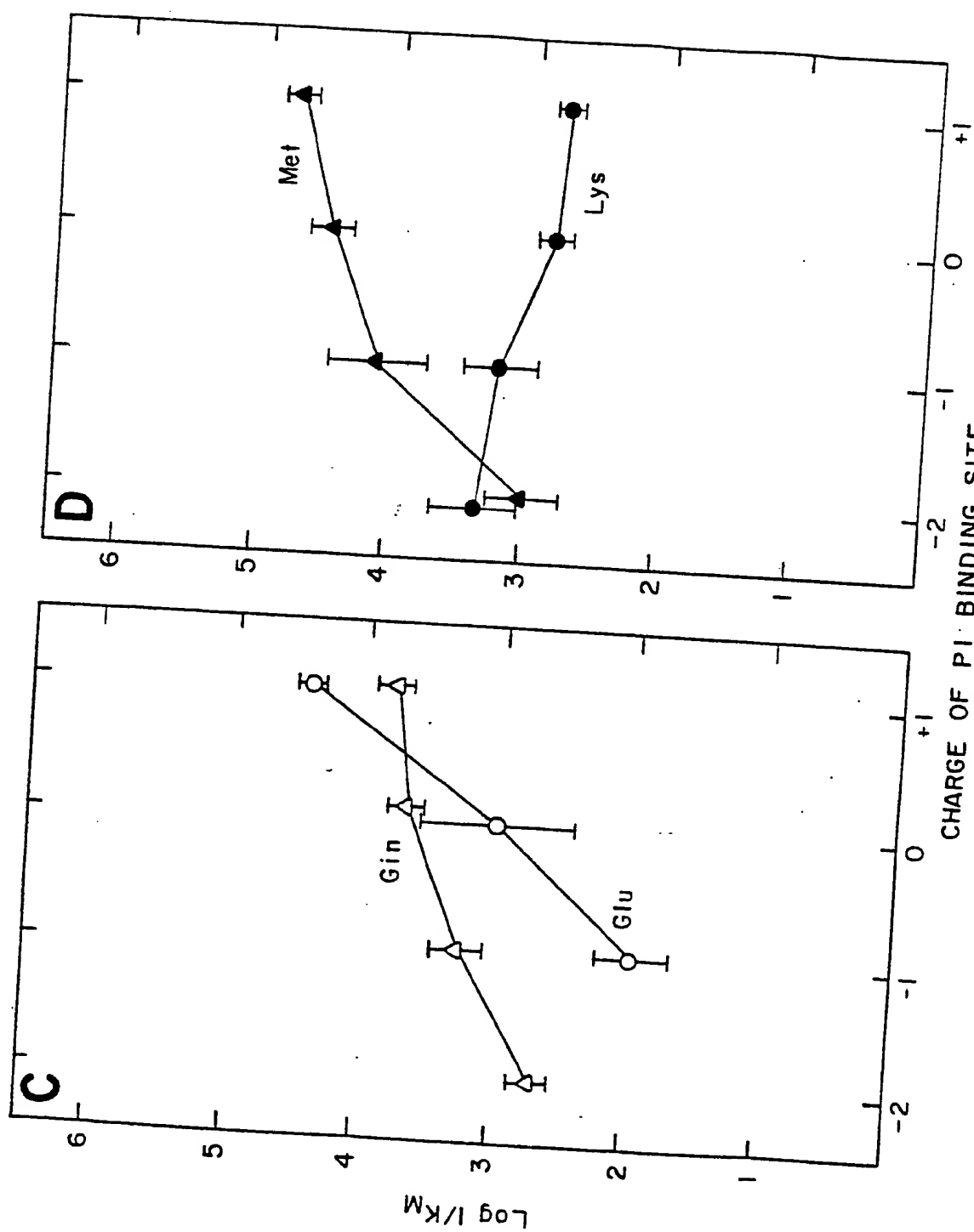


FIG. -28

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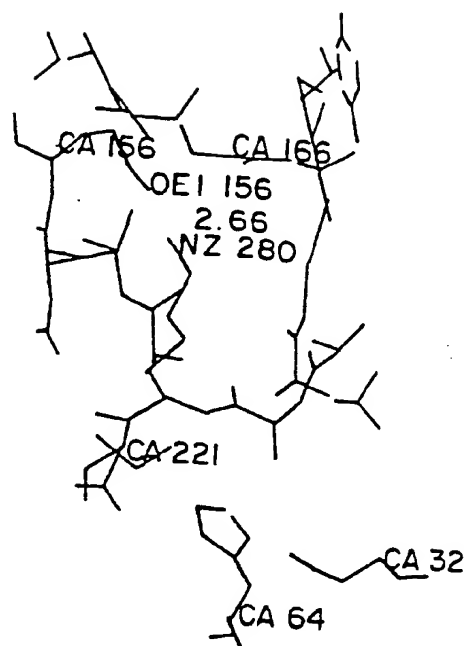
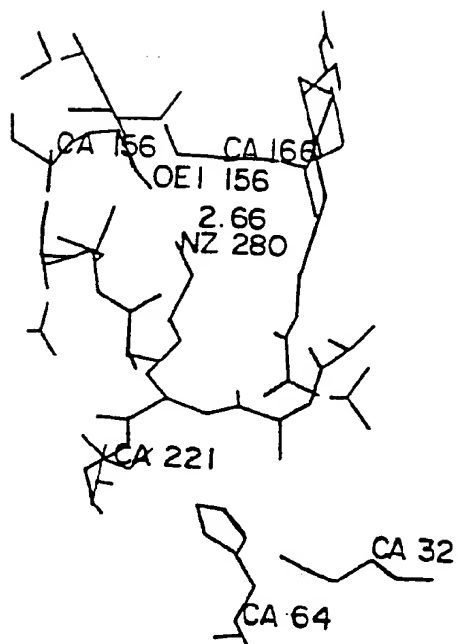


FIG. — 29A

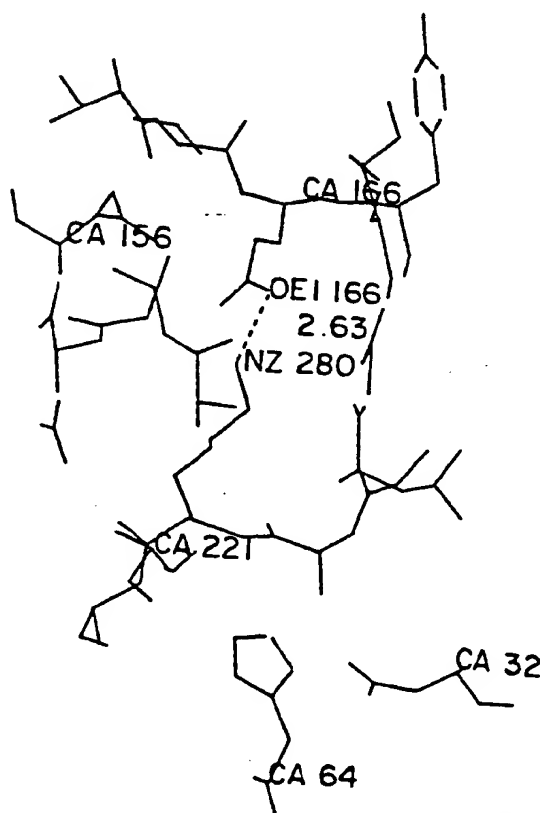
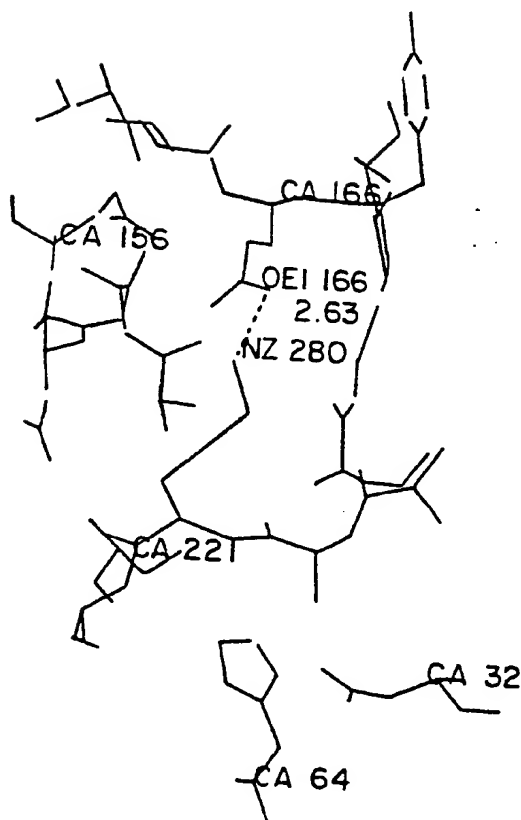


FIG. — 29B

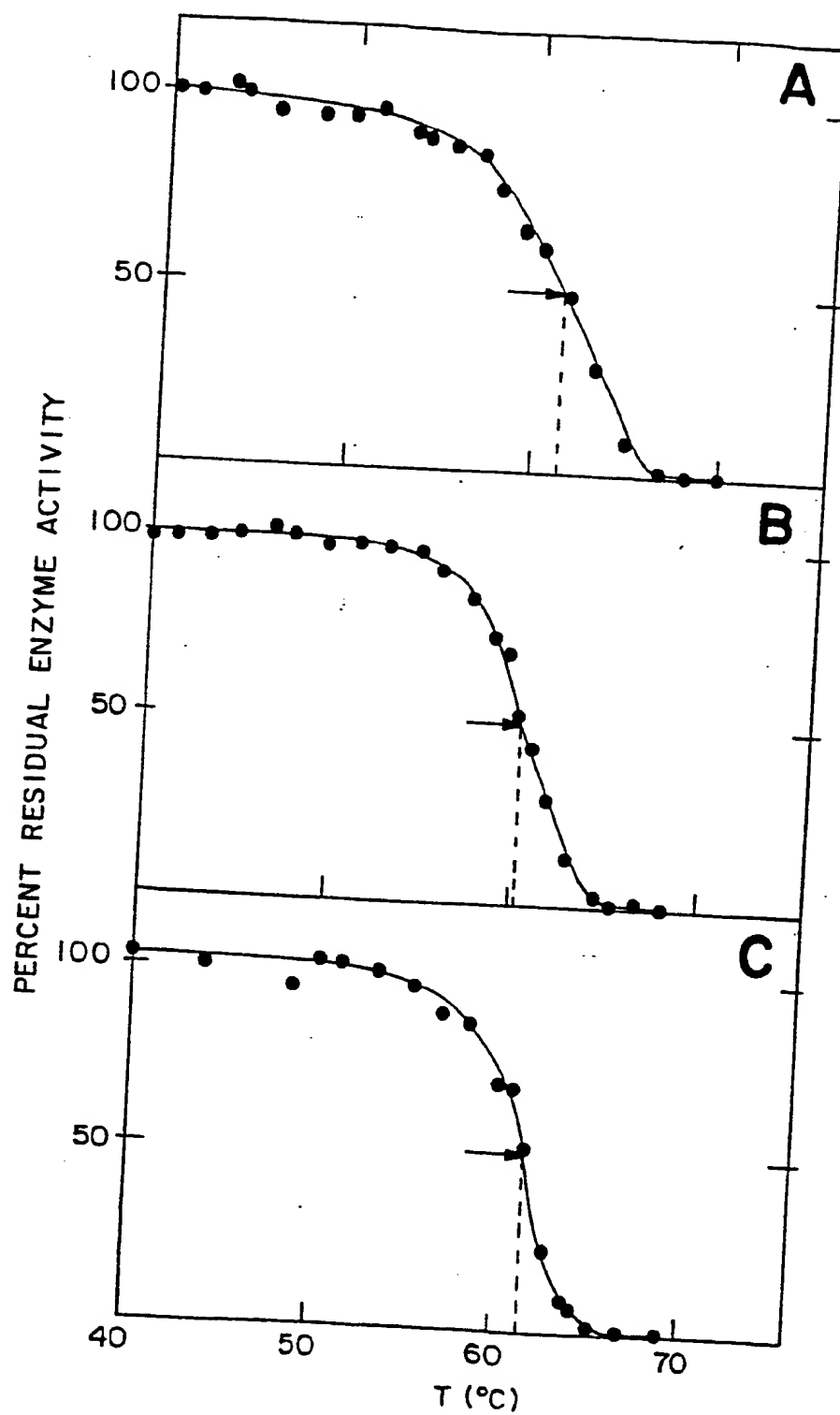


FIG.-30

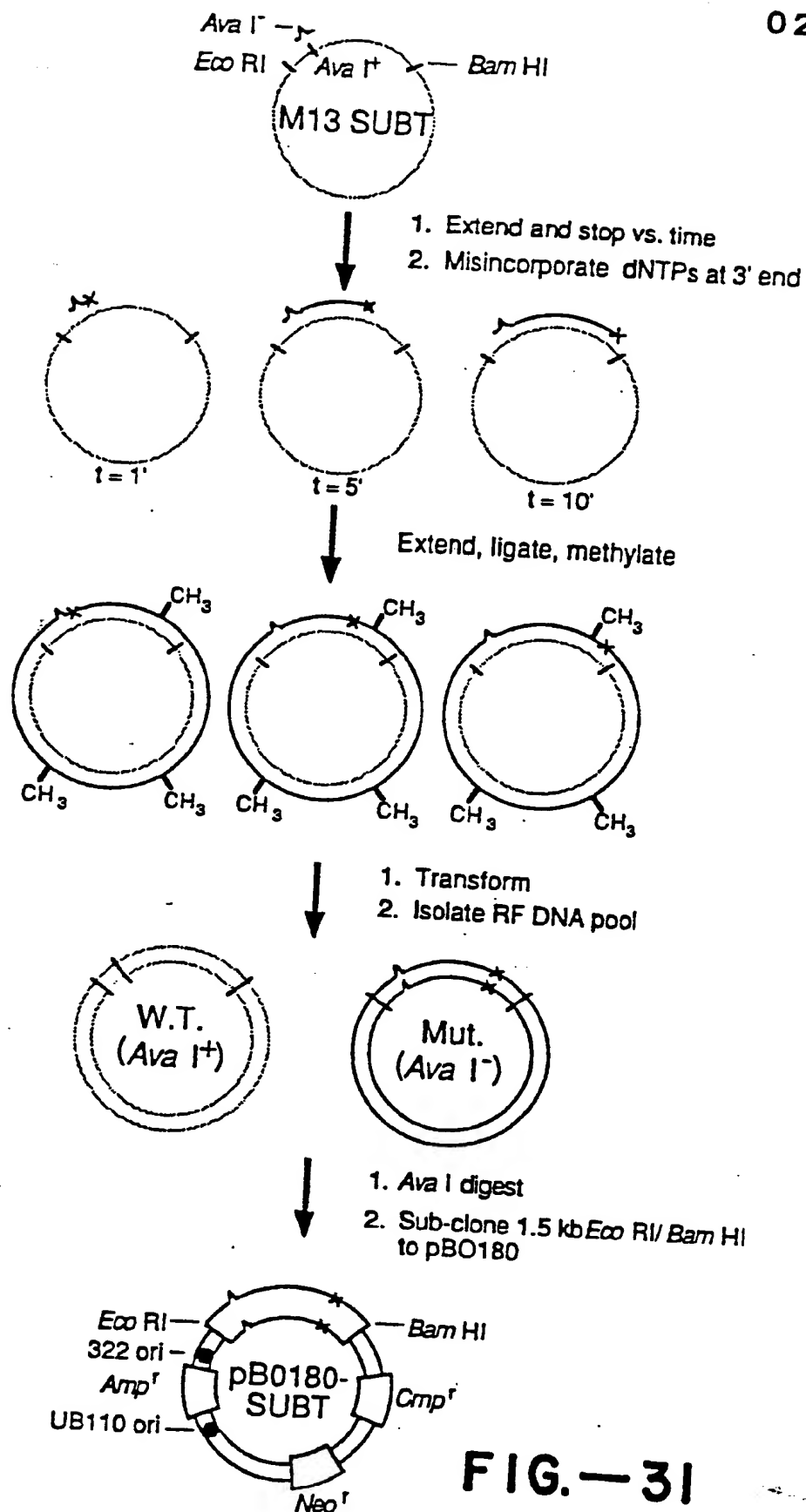


FIG.—31

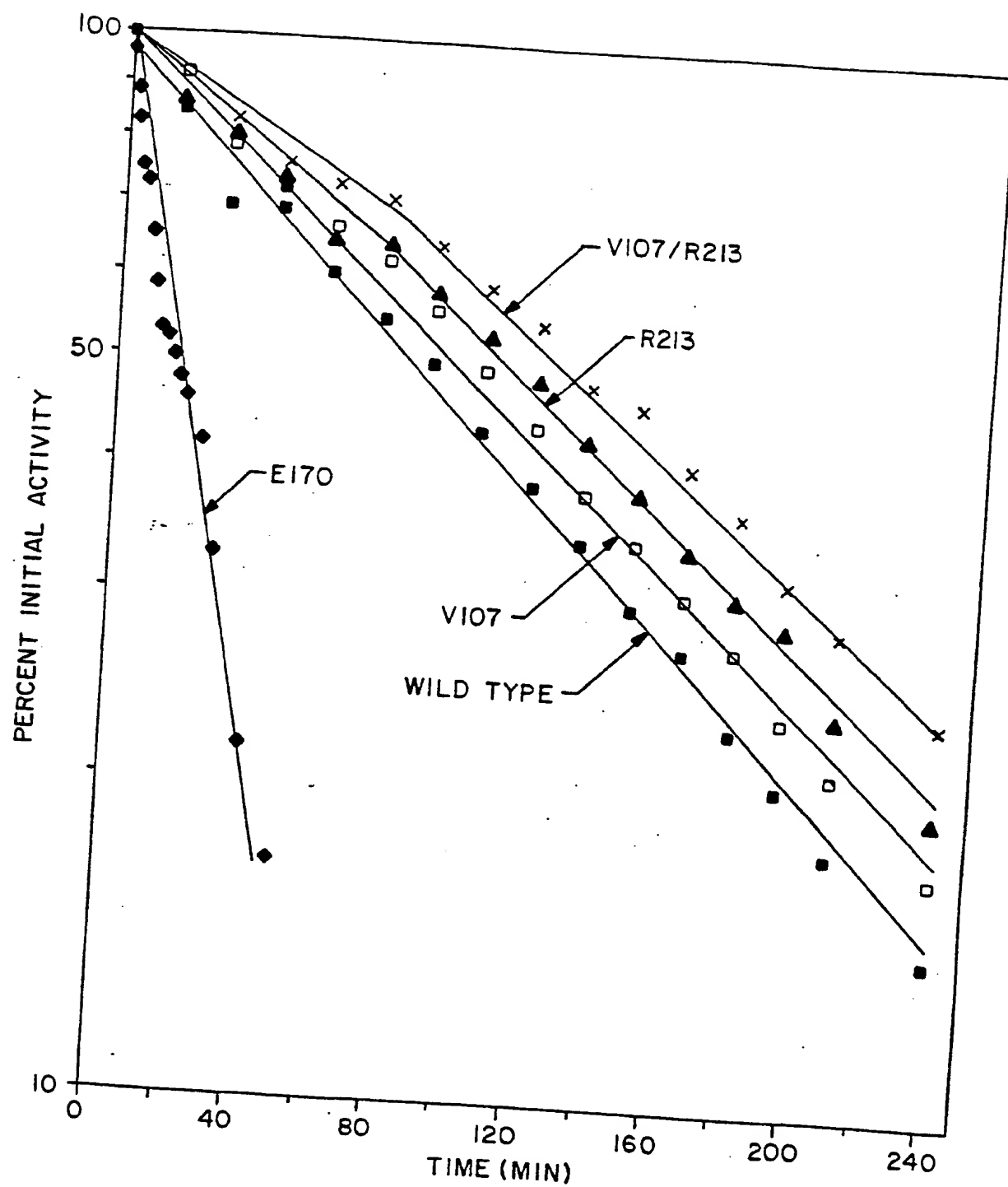


FIG.-32

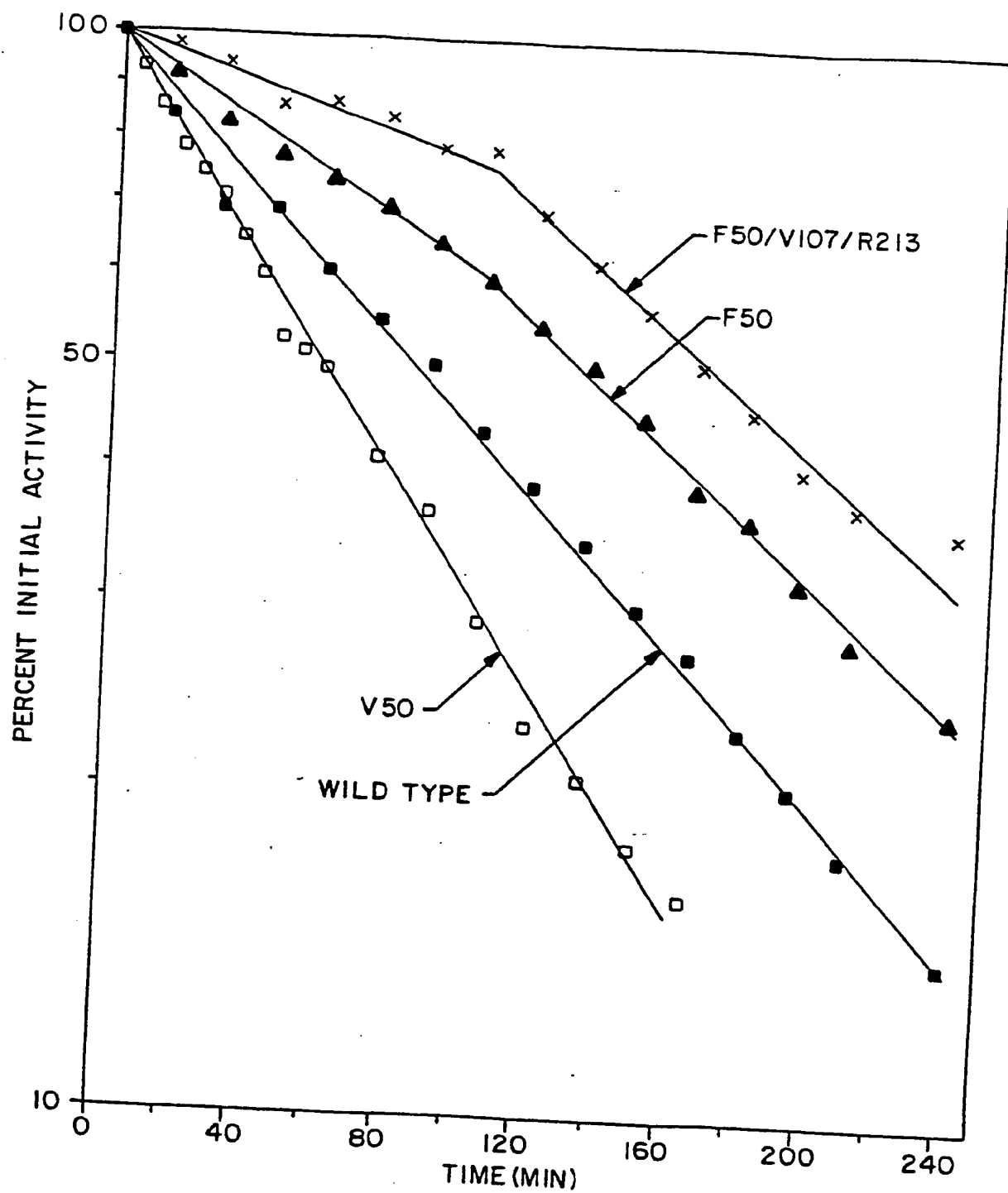


FIG.-33

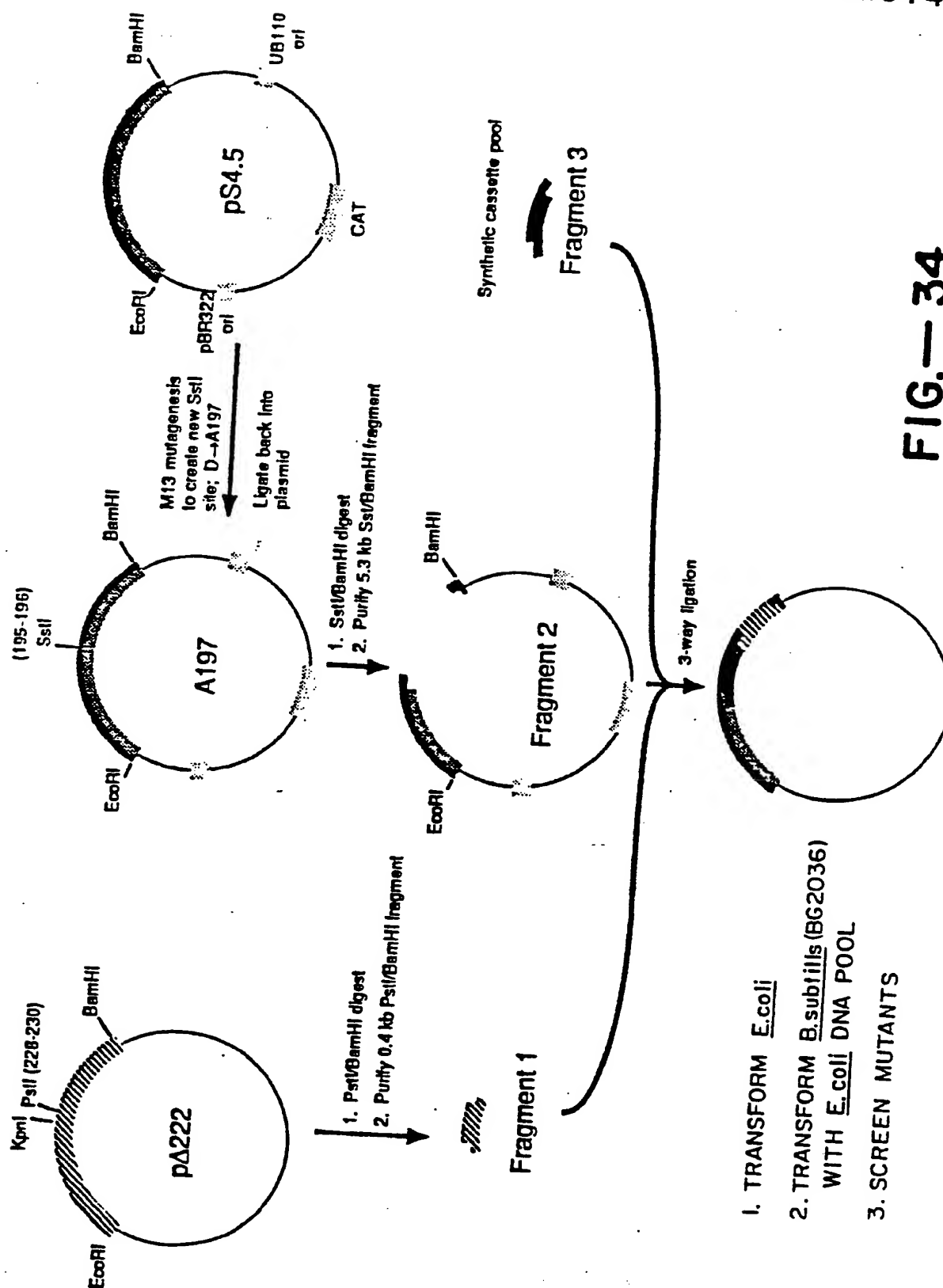


FIG.— 34

0251446

195 200
 W.T.A.A.: Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln
 W.T. DNA: GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA
 CTC GAA CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT
 pA222 DNA: GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA
 CTC GAA CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT
 A197 DNA: GAG CTC GCA GTC ATG GCA CCT GGC GTA TCT ATC CAA
 CTC GAG CGT CAG TAC CGT GGA CCG CAT AGA TAG GTT
 SstI
 Fragments from
 pA222 and A197
 cut w/ PstI, SstI:
 GAG-CT
 Cp
 *
 pA222, A197
 cut & ligated
 w/ oligodeoxy-
 nucleotide pools:
GAG CTC GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA
CTC GAG CTA CAG TAC CGT GGA CCG CAT AGA TAG GTT
 SstI

207 210 218
 W.T.A.A.: Ser Thr Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn
 W.T. DNA: AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC
 TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC ATG TTG
 pA222 DNA: AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC
 TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC ATG TTG
 A197 DNA: AGC ACG CTT CCT GGA AAC AAA TAC GGG GCG TAC AAC
 TCG TGC GAA GGA CCT TTG TTT ATG CCC CGC ATG TTG
 Fragments from
 pA222 and A197
 cut w/ PstI, SstI:
 * *
AGC ACG CTT CCC GGG AAC AAA TAC GGG GCG TAC AAC
TCG TGC GAA GGG CCC TTG TTT ATG CCC CGC ATG TTG
 SmaI

219 220 230
 W.T.A.A.: Gly Thr Ser Met Ala Ser Pro His Val Ala Gly Ala
 W.T. DNA: GGT ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG-3'
 CCA TGC AGT TAC CGT AGA GGC GTG CAA CGG CCT CGC-5'
 pA222 DNA: GGT ACC TCA-----CG CAC GCT GCA GGA GCG-3'
 CCA TGG AGT-----GC GTG CGA CGT CCT CGC-5'
 A197 DNA: KpnI
 PstI
 GGT ACG TCA ATG GCA TCT CCG CAC GTT GCC GGA GCG-3'
 CCA TGG AGT TAC CGT AGA GGC GTG CAA GTG CCT CGC-5'
 PGGG GCG-3'
 A CGT CCT CGC-5'
 *
 Fragments from
 pA222 and A197
 cut w/ PstI, SstI:
 pA222, A197
 cut & ligated
 w/ oligodeoxy-
 nucleotide pools:
GGT ACC TCA ATG GCA TCT CCG CAC GTT GCA GGA GCG-3'
CCA TGG AGT TAC CGT AGA GGC GTG CAA CGT CCT CGC-5'
 KpnI
 PstI destroyed

Oligodeoxynucleotide pools synthesized with 2% contaminating nucleotides in each cycle to give
 -15% of pool with 0 mutations, -28% of pool with single mutations, and
 -57% of pool with 2 or more mutations, according to the general formula $f = \frac{\mu^n}{n!} e^{-\mu}$.

FIG.—35

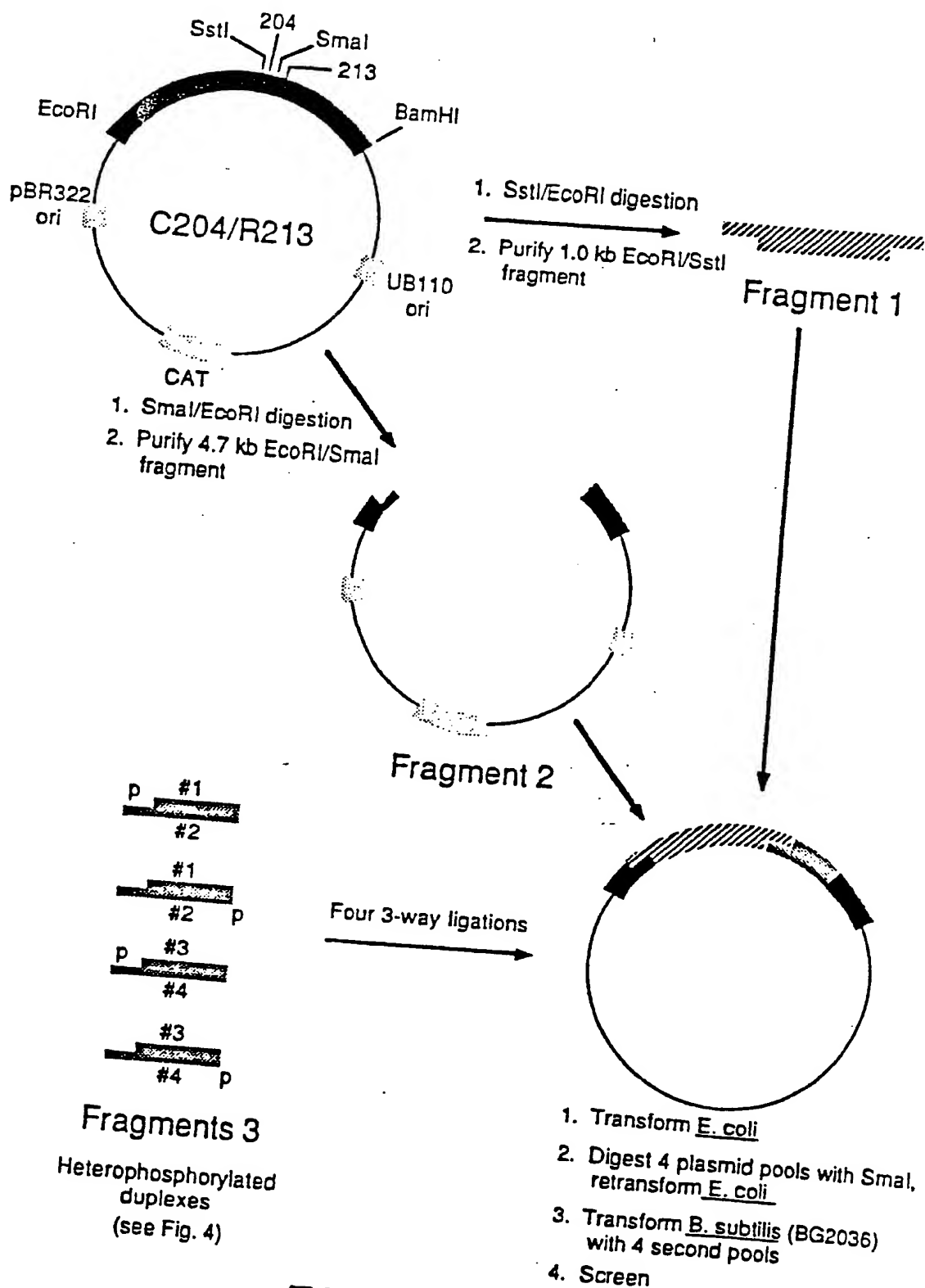


FIG.—36

Wild type A.A.:
 195 Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Glu Ser Thr Leu Pro Gly Asn Lys 210 213
 Wild type DNA:
 5'-GAG CTT GAT GTC ATG GCA CCT GGC GTA TCT ATC CAA AGC ACG CTT CCT GGA AAC AAA-3'
 3'-CTC GAA CTA CAG TAC TAC CGT GGA CCG CAT AGA TAG GTT TCG TGC GAA GGA CCT TTG TTT-5'

C204/R213 DNA:
 5'-GAG CTC GAT GTC ATG GCA CCT GGC GTA TGT ATC CAA AGC ACG CTT CCC GGG AAC AGA-3'
 3'-CTC GAG CTA CAG TAC CGT GGA CCG CAT ACA TAG GTT TCG TGC GAA GGG CCC TTG TCT-5'
 SstI SmaI

C204/R213 cut
 with SstI and SmaI:
 5'-GAG CT
 3'-C

GGG AAC AGA-3'
 CCC TTG TCT-5'

C204/R213 cut and
 ligated with oligo-
 deoxynucleotide pools:

5'-GAG CTC GAT CTC ATG GCA CCT GGC GTA ATC CAG TCG ACG CTT CCT GGG AAC AGA-3'
 3'-CTC GAG CTA CAG TAC CGT GGA CCG CAT TAG GTC AGC TGC GAA GGA CCC TTG TCT-5'
 SstI SmaI

W, R, R, or G ← NGG or NCC → S, P, T or A
 Stop, Y, H, Q, N, K, D or E ← [G]_{TN} or [C]_{TN} → L, F, I, V or M

11 12 13 14

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FIG.—37

①2

EUROPEAN PATENT APPLICATION

②1 Application number: 87303761.8

②2 Date of filing: 28.04.87

⑤1 Int. Cl.³: **C 12 N 15/00**
C 12 N 9/54, C 12 N 1/00

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06.04.87 US 35652

④3 Date of publication of application:
07.01.88 Bulletin 88/1

⑧8 Date of deferred publication of search report: 23.11.88

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AT BE CH DE ES FR GB GR IT LI LU NL SE

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⑤4 Non-human Carbonyl hydrolase mutants, DNA sequences and vectors encoding same and hosts transformed with said vectors.

⑤7 Novel carbonyl hydrolase mutants derived from the amino acid sequence of naturally-occurring or recombinant non-human carbonyl hydrolases and DNA sequences encoding the same. The mutant carbonyl hydrolases, in general, are obtained by *in vitro* modification of a precursor DNA sequence encoding the naturally-occurring or recombinant carbonyl hydrolase to encode the substitution, insertion or deletion of one or more amino acids in the amino acid sequence of a precursor carbonyl hydrolase. Such mutants have one or more properties which are different than the same property of the precursor hydrolase.

EP 0 251 446 A3



European Patent
Office

EUROPEAN SEARCH REPORT

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Application Number

EP 87 30 3761

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X,D	EP-A-0 130 756 (GENENTECH INC.) * Claims 11,12,15,26,28 *	2,3,6-10	C 12 N 15/00 C 12 N 9/54 C 12 N 1/00
X,O	ABSTRACTS OF THE 190TH AMERICAN CHEMICAL SOCIETY NATIONAL MEETING, vol. 190, 1985, page 23, no. 47; R.R. BOTT et al.: "Protein engineering of subtilisin" * Abstract *	1-3,8-10	
X,O	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPL., vol. 0, no. 10, part A, 1986, page 271, no. E101, SYMPOSIUM ON PROTEASES IN BIOLOGICAL CONTROL AND BIOTECHNOLOGY, 15th ANNUAL UCLA MEETING ON MOLECULAR AND CELLULAR BIOLOGY, Los Angeles, CA., 9th-15th February 1986; P. BRYAN et al.: "Protein engineering of subtilisin proteases of enhanced stability" * Abstract *	1-5,8-10	
A	WORLD BIOTECH. REPORT, vol. 2, 1985, pages 51-59, Online Publications, Pinner, GB; R. BOTT: "Modeling & crystallographic analysis of site-specific mutants of subtilisin"	4,8-10	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 12 P
X	IDEM	6,8-10	
P,X O	JOURNAL OF CELLULAR BIOCHEMISTRY SUPPL., vol. 0, no. 11, part C, 1987, page 200, no. N024, New York, US; D.A. ESTELL et al.: "Tailoring enzymatic properties through multiple mutations" * Abstract *	2,3,4,5,8-10	
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 09-08-1988	Examiner VAN PUTTEN A.J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

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Office

EUROPEAN SEARCH REPORT

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Page 2

Application Number

EP 87 30 3761

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, vol. 84, March 1987, pages 1219-1223, Washington, D.C., US; J.A. WELLS et al.: "Designing substrate specificity by protein engineering of electrostatic interactions" * Paragraph "Materials and methods" *	2-10	
P,X	BIOCHEMISTRY, vol. 26, no. 8, April 1987, pages 2077-2082, American Chemical Society, Washington, D.C., US; M.W. PANTOLIANO et al.: "Protein engineering of subtilisin BPN': enhanced stabilization through the introduction of two cysteines to form a disulfide bond" * Abstract *	2-10	
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE USA, vol. 83, June 1986, pages 3743-3745, Washington, D.C., US; P. BRYAN et al.: "Site-directed mutagenesis and the role of the oxyanion hole in subtilisin" * Abstract *	3,8-10	
E	WO-A-8 704 461 (AMGEN) * Page 6, line 1 - page 8, line 6 *	1,2,3,6-10	
X	NATURE, vol. 318, 28th November 1985, pages 375-376, London, GB; P.G. THOMAS et al.: "Tailoring the pH dependence of enzyme catalysis using protein engineering" * Page 375, column 1, lines 27-30; table 1 *	3,8-10	TECHNICAL FIELDS SEARCHED (Int. Cl.4)

The present search report has been drawn up for all claims.

Place of search
THE HAGUE

Date of completion of the search
09-08-1988

Examiner
VAN PUTTEN A.J.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document



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EUROPEAN SEARCH REPORT

Page **0251446**

Application Number

EP 87 30 3761

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
E	WO-A-8 705 050 (GENEX CORP.) * Page 18 *	1,2,3,8 -10	
A	--- JOURNAL OF BACTERIOLOGY, vol. 158, no. 2, May 1984, pages 411-418, American Society for Microbiology, Washington, D.C., US; M.L. STAHL et al.: "Replacement of the Bacillus subtilis subtilisin structural gene with an in vitro-derived deletion mutation"		
A	--- NUCLEIC ACIDS RESEARCH, vol. 11, no. 22, November 1983, pages 7911-7925, IRL Press Ltd, Cambridge, GB; J.A. WELLS et al.: "Cloning, sequencing, and secretion of Bacillus amyloliquefaciens subtilisin in Bacillus subtilis"		
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 09-08-1988	Examiner VAN PUTTEN A.J.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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